The impact of arbuscular mycorrhizal fungal inoculation on the growth and nutrition of agricultural plant species

Binh Tran Thi Thanh

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School of Agriculture Food and Wine

The University of Adelaide

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Thesis abstract

Arbuscular mycorrhizas (AM) can improve the nutrition of plants by increasing the uptake of nutrients, including phosphorus (P), zinc (Zn), and other micronutrients. It is for this reason that AM are often cited as having an important role to plant in enhancing the yield and mineral nutrition of food crops and helping to meet the demands of a growing world population, especially in changing climate. However, interactions between the arbuscular mycorrhizal fungi (AMF), plant, and environmental factors are complex and highly variable. Therefore, an understanding of the effect of forming AM on the growth, yield, and nutrition of agriculturally important crops is critical in order to design a sustainable farming system that can best harness the benefits of AM.

In this thesis, I focused on exploring the impact of single AMF species *Rhizophagus irregularis* on the growth and nutrition of a range of important crop and pasture species, and different crop genotypes. Then, I further assessed the impact of AM on plant nutrition by studying the bioavailability of Zn and iron (Fe) in durum wheat grain for the purpose of human nutrition. In *Chapter two*, the results demonstrated that arbuscular mycorrhiza formation in diverse host plant species resulted in different responses in root colonisation, growth, and nutrition. Furthermore, plant species was a much stronger driver than colonisation by arbuscular mycorrhizal fungus, especially the plant ionome. However, the formation of AM improved uptake of mineral nutrients such as P, Zn, and Cu of most plant species included in the experiment. The results of *Chapter three* showed that AM increased the phytic acid (PA) concentration of durum wheat grain, which has important implications for estimating the bioavailability of Zn and Fe. In *Chapter four*, I reported on the effects of forming AM on a group of ten diverse durum wheat genotypes. In this experiment, plant genotype had an important role in controlling the responses of plants to AM in terms of yield and nutrition. Additionally, AM increased the bioavailability Zn and Fe in durum wheat grain of some genotypes, but not all.

In addition to exploring impacts of plant identity on arbuscular mycorrhiza formation and functioning, the impact of soil P and Zn nutrient addition, on AM was also studied. Soil P addition had a strong impact on both plant growth and nutrition. It not only improved the plant yield and but also had less obvious effects on AM such as suppressing root colonisation, reducing the concentration of grain Zn and Fe, as well as

increasing PA concentration. In contrast, while soil Zn addition was not found to have significant effects on the growth response of both *Medicago truncatula* and durum wheat to AM of my study, it enhanced the bioavailability of Zn in durum wheat grain. Furthermore, through employing high-throughput phenotyping technology, in *Chapter five*, I found that AM can still positively affect the growth of plants even in high soil P conditions; a response that was not evident in the final harvest. Furthermore, the effect of AM on the plants' growth changed over the life of the plant. This work highlighted the value of phenotyping approaches to the study of impacts of forming AM over the life of a plant as well as at a final harvest.

In conclusion, the impact of AM on plant growth and nutrition is highly variable and context-dependent; there are many factors including plant species and genotypes, soil P and Zn availability and also temporal effects to consider. Therefore, it is important to discover the particular conditions where AM can benefit plants in practical agricultural systems, in both growth and nutrition of specific plants species/genotypes. The effect of AM on PA on the cereals grain is another important factor to consider in the context of human nutritional quality in cereal crops.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Chapter 3

Table 1 ANOVA summary table including degree of freedom (*df*), F statistic (*F*), and probabilities (*P*), for all variables. Factors in the analysis were soil Zn concentration for one-way ANOVA; soil Zn concentration and AMF inoculation for two-way ANOVA. Significant probabilities are highlighted with boldface.

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Thesis overview

This thesis is presented as a series of journal manuscripts and articles which have already published or prepared to be submitted for publication. The body of work examines the relationships between AM, soil P/Zn on the growth and mineral nutrition of different agriculturally important crop and pasture plant species. This included four glasshouse-based experiments.

In order to provide an overview of the context to the overall project, in *Chapter one,* I gave a short literature review which was prepared at the start of this project. Because the literature related to each researching paper was written in the introduction section of each chapter, they are not presented in this literature review. The objectives of the whole project are also presented in this chapter.

In *Chapter two, I* present my first paper. In this paper, I analysed the responses in growth and nutrition of fifteen crops and pasture plant species to the inoculation of a single AMF species. It was published in the Functional Plant Biology, in 2019.

From the result of *Chapter two,* durum wheat was chosen for further examining their responses in growth and nutritional quality especially in the food parts. The finding was presented in the *Chapter three* and published in the journal of Mycorrhiza, in 2019.

The results from *Chapter three* indicated significant effect of arbuscular mycorrhizal fungal inoculation on phytic acid concentration of durum wheat grain, which underpin the work presented in *Chapter four*. In *Chapter four* I present the work in which, I analysed phytic acid content and the mineral nutrition of 101 genotypes of durum wheat and determined the effect of arbuscular mycorrhizal fungal inoculation and phosphorus fertiliser on the productivity and nutritional value of the grain of ten chosen durum wheat genotypes.

Chapter five focuses on the temporal growth response of plant to arbuscular mycorrhizal fungal inoculation and zinc fertiliser using the high-throughput phenotyping system, which was published in the journal of Plants, People, Planet, 2020.

The thesis concludes by *Chapter 6* with a general discussion of the main findings and relationships found herein and give recommendations for future work.

Chapter 1 - Introduction

Literature review

Arbuscular mycorrhizal fungi

What are arbuscular mycorrhizas?

Arbuscular mycorrhizal fungi (AMF) belong to the phylum *Glomeromycota* (Schüβler et al. 2001). These fungi can form obligate symbiotic relationships with plant roots (Smith and Read 2008). The resulting associations, which are called arbuscular mycorrhizas (AM), include the plant roots, and fungal structures with within the roots (e.g. arbuscules, vesicles, hyphal coils), and the surrounding soil (e.g. extra-radical hyphae and spores) (Smith and Read 2008). While an estimated 85% of flowering plant species form mycorrhizas of one type or another, the AM are the most common, accounting for 80% out of the 85% (Brundrett 2009).

Arbuscular mycorrhizas are formed by many important cereal crops (e.g rice, maize, barley and wheat), grain and pasture legumes, vegetables (e.g. tomato, lettuce and cucumber), and fruit trees (e.g. peach, citrus, grape) (Baslam et al. 2013; Baum et al. 2015; Ercoli et al. 2017; Giovannetti and Avio 2002; Harikumar 2017; Ramírez-Flores et al. 2017). In this symbiosis, fungi provide mineral nutrients for plants from the soil such as phosphorus (P), nitrogen (N), copper (Cu), iron (Fe), potassium (K), zinc (Zn), calcium (Ca) and sulphur (S), and can promote plant growth. In return, the AMF receive a supply of carbon (C) from the plants to support activities such as nutrient acquisition, vegetative growth, and spore production (Smith and Smith 2011). It is (largely) for the potential nutritional benefits (to plants) of forming AM, that there has a great interest in the role of AM in agricultural systems.

The nutritional benefits of AM

Arbuscular mycorrhizal fungi increase the pool of nutrients available to plants and can therefore increase the host plants' acquisition of nutrient such as inorganic P, N, Cu, Fe and Zn in the low nutrient soils (Giovannetti et al. 2001). These nutrients have a generally low mobility in the soil, and so often form depletion zones around roots (Marschner 2012). The capacity of AMF to extend beyond these depletion zones is an important aspect of how forming AM can help improve plant nutrient uptake. In mycorrhizal plants, most immobile soil nutrients can be uptake through two

pathways: the plant pathways that directly uptake via the plant root systems, and the mycorrhizal pathway that indirectly uptake via the external hyphae systems and then transfer to plant roots(Watts-Williams et al. 2015). Radioisotope tracing techniques have been used widely to demonstrate the contribution of the mycorrhizal pathway to the uptake of various nutrients including P and Zn (Hodge and Fitter 2010; Smith et al. 2004; Watts-Williams et al. 2015).

The positive effects of AM on plant P uptake are substantial and well-studied. As an essential plant macronutrient, crops have specific minimum P requirements for their growth and productivity (Barry and Miller 1989; Grant et al. 2001). For example, total P uptake for maximum yield by wheat, rice and sorghum were 23, 20, and 38 kg ha^{-1}, respectively (Obaid-ur-Rehman et al. 2007). In plants, P deficiency may inhibit leaf expansion, decrease the number of leaves, flowers, restrict seed formation (Fredeen et al. 1989; Lynch et al. 1991) and cause imbalanced root/shoot ratio, which can consequently lead to increased incidence of plant disease (Anuradha and Narayanan 1991; Hawkesford et al. 2012; Lambers et al. 2010). Phosphorus is widely cited as the second most limiting nutrient (after N) for crop growth and production worldwide (Aerts and Chapin 1999; Lambers et al. 2011), due to its low availability and mobility (via depletion zones) in the soil. With the ability of AMF to increase the volume of soil that can be explored (e.g. due to distance or pore size), they have the potential to increase the uptake of nutrients such as P (Bertolazi et al. 2018). Furthermore, AMF can also produce hydrolytic enzymes that can release P in organic P compounds into the soil solution, thereby helping to enhance plant P uptake under P limited conditions (Koide and Kabir 2000). This may result in a significant increase in plant growth, productivity and P concentrations in plant tissues (Smith et al. 2011). However, a positive mycorrhizal growth response is not always correlated with the amount of P being transferred to the plant by AM. For example, Smith et al. (2003) found that even when the overall growth of host plant or P uptake was not promoted by AM, the majority proportion of total P acquired can be taken up via the external AM hyphae in soil. It is however clear that the most significant benefits of forming AM are observed when P concentrations in the soil are low, and benefits tend to diminish with increasing soil (available) P supply (Lekberg and Koide 2005). Indeed, mycorrhizal growth responses may become neutral or even negative, when P is in plentiful supply, because the cost of C supply for AMF activities negate the benefit of mineral nutrients uptake (Smith and Read 2008).

A positive effect of AM on N uptake has also been revealed; radioisotope labelling experiments have been demonstrated that a significant proportion of N can be absorbed and transferred to plants by AM (Hodge and Fitter 2010; Leigh et al. 2009; Tanaka and Yano 2005). Due to the relatively higher mobility of N in the soil (especially nitrate), the impact of forming AM on plant N nutrition is less significant than that for P (Smith and Read 2008). In comparing P to N uptake, the mycorrhizal pathway can deliver up to 100% of plant P and 20% of plant N (Cavagnaro et al. 2015; Smith et al. 2003).

The external hyphae of AMF can increase plant uptake of micronutrients, such as Zn, Fe and Cu in low nutrient condition; however, the mechanisms involved are less well understood (Clark and Zeto 2000; Smith and Read 2008). These heavy metals are required by plants, in low concentrations, to function as enzyme cofactor or maintain plant physiological processes, however, high concentrations can inhibit plant growth (Khan et al. 2015). The mycorrhizal pathway includes the external hyphae uptake, the long-distance translocation inside the hyphal system, and the transport across the plant-fungal interface, the peri-arbuscular membrane, by specialised proteins. While the external hyphae uptake of some nutrients have been proposed well, the two later mechanisms remain undiscovered. While some the arbuscular mycorrhizal specific transporters in *Rhizophagus irregularis* have been discovered, such as RiCTR1 and RiCTR3 for Cu, RiZRT1 for Zn and RiFTR1 for Fe (Tamayo et al. 2014), the functioning mechanism of these transporter is remain unknown. The role of the phosphate transporter MtPT4 in *Medicago truncatula* has been well established to facilitate the movement of Pi across the AM fungal-plant interface on the peri-arbuscular membrane (Harrison et al. 2002; Javot et al. 2007). In addition, studies found that AM induced changes in metal transporters genes in plants (Gomez et al. 2009). For example, in *Medicago truncatula*, Zn transporter genes of MtZIP6 was found up-regulated at low soil Zn (Watts-Williams et al. 2017). It was proposed that the metals transporters localise on the peri-arbuscular membrane facilitating the metals transportation into plants (Ferrol et al. 2016). However, these understanding of molecular basis of these heavy metal nutrient uptake through the mycorrhizal pathway is still at the starting point.

In terms of Zn, arbuscular mycorrhizal fungal inoculation is beneficial to plants under both deficient and toxic condition of soil Zn (Watts-Williams et al. 2013). When Zn in soil is deficient, AM increase Zn uptake thus increase plant tissue

concentration of Zn (Pellegrino et al. 2015). It is estimated that up to 24% of shoot Zn of the mycorrhizal tomato plants can be supplied by AM and this decreases as the Zn availability in soil increases (Watts-Williams et al. 2015). When Zn in soil under toxic conditions, AM can protect plants from excessive Zn uptake by reducing shoot Zn concentrations and increase shoot biomass relative to non-mycorrhizal control plants in red clover (Chen et al. 2003). This may be due to the ability of absorbing and storing metal ions in fungal structures (e.g. vacuoles and spores) of the arbuscular mycorrhizal external hyphae (Gong and Tian 2019). The understanding about the effects and underlying mechanisms of AM on the uptake via the mycorrhizal pathway of other metal nutrients are limited and require further investigation (Ferrol et al. 2016).

Arbuscular mycorrhizal fungi have also been found to alleviate the effects of environmental stress by interfering the balance of the host plant's phytohormones (Baum et al. 2015). These stressors may include drought (Augé 2000), salinity (Porcel et al. 2012), heavy metal contamination (Garg and Chandel 2011), and acidic soil (Rouphael et al. 2010). Other beneficial effects of forming AM include an increase resistance of plants to pathogens (e.g. nematodes) (Baum et al. 2015). The formation of AM can enhance soil aggregation and improve soil structure, thereby reducing erosion risk, improving water movement, and reducing the risk of soil nutrient loss (Cavagnaro et al. 2015; Pellegrino et al. 2015).

The aforementioned functions of AM may have beneficial or detrimental effects on plant growth (biomass) and yield, but also change the tissue nutrient concentration and content (Antunes et al. 2012; Giovannetti et al. 2012). However, there is relatively little information about whether AM increase yields and nutritional value of the edible portions of plants (i.e. fruits, seeds/grains, tubers). Such information is essential in determining the true potential of AM to improve human nutrition and will be discussed further below.

Food security and arbuscular mycorrhizas

Food security

There is a great food demand to supply for the rapidly growing human population. It is estimated that the world population between 2030 and 2040 will be about 8 billion people with 5.5×10^9 metric tons of food required (Vance 2001). Furthermore, the deficiencies of micronutrients, especially vitamins and minerals such as Vitamin A,

Fe, Zn, Cu, Mg, iodine, also known as the 'hidden hunger', were found in one of every three people worldwide (FAO 2013). Micronutrient deficiencies can lead to mental impairment, poor health, low productivity, and even death in humans (White and Broadley 2009). It may be caused by foods in the daily diet, which contain adequate calories, but lack essential micronutrients for human requirements. Therefore, producing adequate food with sufficient nutrient content for the rapidly growing world population is imperative if we are to achieve global food security. As arable land is finite, there is increasing urgency to improve the crop yield and increase the concentration and bioavailability of mineral elements in food, also known as biofortification, in a sustainable manner. Because AM may enhance plant nutrient assimilation, it has been suggested that they may have an important role to play in improving the nutritional value of crops, including biofortification (Antunes et al. 2012), which will now be discussed.

The potential of arbuscular mycorrhizas in biofortification of crops

It has been well-documented in the literature that AMF have an important role in boosting nutrient levels particularly N, P, Cu, Mg, Fe and Zn in plants based on the ability to access minerals, most importantly in nutrient stressed environments (Baslam et al. 2013; Baslam et al. 2011; Baum et al. 2015; Hart et al. 2015b; Hu et al. 2013; Salvioli et al. 2012; Watts-Williams and Cavagnaro 2012). The responses of the host plant to AM have been found to be highly variable among species of AMF and plants. However, in general, growth improvements and higher minerals concentration in mycorrhizal plants were correlated with better mineral absorption by mycorrhizal roots. Furthermore, the enhanced mineral uptake varies depending on the amount of available minerals in the soil (Hart et al. 2015a). However, this remains to be tested in a range of different crops and AMF species. While there exists much data on the role of AM on the plant's root/shoot tissue nutrient concentrations (especially P), studies that target the food parts of plants (grains, fruits, tubers, etc.) are relatively few in number. This information is vital to assess the true value of AM in human nutrition (Antunes et al. 2012). The study of Giovannetti et al. (2012) which investigated the effect of *Rhizophagus irregularis* on the nutraceutical value and safety of tomato fruits, showed that AM enhanced both growth and mineral nutrient content of tomato plants as well as nutritional value of tomato fruits especially fruit P and Zn contents. Furthermore, Pellegrino et al. (2015) conducted a meta-analysis

of 38 field trials of wheat, which considered 333 observations of wheat's responses for plant biomass, grain yield, nutrient acquisition. The results demonstrated that the grain yield was positively correlated with arbuscular mycorrhizal colonisation. In addition, field arbuscular mycorrhizal fungal inoculation increased not only the tissue N, P concentration but also the grain N and Zn content. To sum up, these studies showed that AMF is the promising resource to improve food quantity and quality, but that there is a dearth of such studies.

Impact of plant identity on mycorrhizal responses of growth and nutrition

Diverse crops responded diversely to arbuscular mycorrhizal fungal colonisation and these responses varied from positive, neutral to negative (Hoeksema et al. 2010). Based on biomass and nutritional responses, some studies have reported that C_4 grasses have a higher positive response to mycorrhizal inoculation compared to C_3 grasses (Hoeksema et al. 2010; Wilson and Hartnett 1998). Furthermore, a meta-analysis of Hoeksema et al. (2010) also found that plants with N-fixing bacterial symbionts were generally less responsive to AM compared to those without N-fixing symbionts. It has been explained that the soil P concentrations in those analysed studies were relatively high and/or the expense of plant-C required to maintain the two symbionts was high (Bethlenfalvay et al. 1985). A review highlighted that root morphological characteristics such as length, dry weight, root hair length, density of root hairs, were negatively correlated with the growth responses of plant by AM (Tawaraya 2003). This is in agreement with many studies that emphasise the extremely important role of root morphology in determining mycorrhizal responsiveness (Hetrick et al. 1991; Hetrick et al. 1990; Smith and Smith 1996). Together these studies indicate that while some generalisations about how different groups of plants might respond to the formation of AM, there is still much to be discovered.

To further complicate the matter of plant identity and arbuscular mycorrhizal responses, there can also be large variation in the growth responses among genotypes of the same crop (Cobb 2016; Smith and Read 2008). This has been reported in many agriculturally important crops and pasture species, such as in sunflower (Turrini et al. 2016), sorghum (Cobb et al. 2016; Watts-Williams et al. 2019b), *Medicago truncatula* (Medicago) (Watts-Williams et al. 2019a), chickpea (Bazghaleh et al. 2018), maize (An et al. 2010) and bread wheat (Hetrick et al. 1995; Zhu et al. 2001).

It has been explained that plants from different genotypes may exude different phytochemicals from their roots, which may be involved in signalling and initiating the formation of AM (Ellouze et al. 2016; Venturi and Keel 2016). Moreover, the distinction in root traits such as length and density among genotypes of plants can also contribute to this variation (Bazghaleh et al. 2018; Kashiwagi et al. 2006). In particular, genotypes with longer and finer root generally benefit less from forming AM (Zangaro et al. 2007). Consequently, genotypes of the same plant species are also diverse in their response to AM in their nutritional response.

The recent study of Cobb (2016) found the significant correlation between total content of such minerals as Fe, P, Mg and Zn of sorghum grain and AM colonisation across all genotypes, however this correlation was not found across genotypes of common bean and cowpea. Furthermore, the formation of AM affected different Medicago genotypes diversely in the plant's water relations (Watts-Williams et al. 2019a). Breeding strategies generally maximize food production and often relate to high water, pesticides and fertilisers used (Lehmann et al. 2012), and some authors have noted that these selective processes may have adverse effects on the plant compatible capacity of the optimal genotypes to AMF (Hetrick et al. 1995; Turrini et al. 2016; Zhu et al. 2001). Because the most beneficial condition of AM is low nutrient soil, for example, the arbuscular mycorrhizal formation may be suppressed in high P soil (Richardson et al. 2011). However, evidence for this concern is still lacking, and the responses of various genotypes from the same plant species to AM, is remained to be widely explored (Lehmann et al. 2012).

In order to design farming systems where AM can improve both production and nutritional value of food crops, it is critical to analyse the responses of crops to arbuscular mycorrhizal fungal inoculation in the yield and nutritional traits. While much is known about the response in the growth and nutrition content of plant tissue to AM (Tawaraya 2003), few studies focused on the nutrient content of edible parts of food crops. Furthermore, the nutrient concentrations of the edible portions of plants are unlikely similar to the tissue nutrient concentrations because the physiological homeostatic mechanisms within the plant are distinct; for example, the nutrient accumulation in seeds is under tighter regulation in compared with in leaves and stems (Grusak and DellaPenna 1999; Rengel et al. 1999). Therefore, it is hard to predict the nutritional quality of crops in response to AM with the currently available literature.

Phytic acid and the effect of arbuscular mycorrhizas on the bioavailability of Zn and Fe in cereal crops

Myo-inositol hexakisphosphate, commonly called phytic acid (PA), is the main storage form of P largely found in grains and seeds (Eagling et al. 2014; Frontela et al. 2009). It can chelate positively charged proteins, amino acid and many nutritionally important minerals such as Zn and Fe forming insoluble phytate-mineral complexes. Because of the lack of phytase enzyme in the human intestine, PA can reduce the bioavailability of these micronutrients during digestion. Therefore, it is the main anti-nutrient compound which leads to Zn and Fe malnutrition in people consuming cereal as staple food. The mineral types and molar ratios of PA to mineral are important to assess the bioavailability of mineral nutrient in food (Weaver and Kannan 2002).

In order to determine the effect of AM on mineral nutrition of food crops, especially Zn and Fe in cereal crops, it is important to examine the effect of AM on the bioavailability of these minerals. However, current studies have focused on the increasing effect of AM on the Zn and/or Fe concentration of cereal grains (Coccina et al. 2019; Ercoli et al. 2017; Lehmann and Rillig 2015; Lehmann et al. 2014; Pellegrino et al. 2015; Zhang et al. 2016) which are not necessarily representing their bioavailability in plant tissues. Therefore, it is important to determine the effect of AM on PA concentrations in cereal crops. However, only few studies have analysed the effect of AM on PA and their conclusions were contradictory (Ma et al. 2019; Ryan et al. 2008; Subramanian et al. 2013). While AM increased PA concentration in the seeds of China jute (Lewis and Koide 1990), they decreased PA concentration in the seeds of maize (Subramanian et al. 2013), and had no effect in the grains of bread wheat (Ma et al. 2019; Ryan et al. 2008). Consequently, the effects of AM on the bioavailabilities of Zn and Fe are also lacking.

High-throughput phenotyping exploring the growth response of plants to arbuscular mycorrhizas

It is well-established that AM may affect the growth of host plant. However, most studies only assess this effect through the dried biomass at final harvest which represents the accumulated effect of arbuscular mycorrhizal colonisation on the plant's growth. In order to explore the underlying mechanism of the interaction among many factors affecting the plant's growth, it is important to analyse the growth

response of the plant to forming AM through time series data. Typical parameters to assess the plant growth over time are shoot biomass or leaf area. However, shoot biomass measuring requires multiple destructive harvests, and leaf area are often measured manually, therefore these methods are time and labour consuming. High-throughput phenotyping (HTP) platform provides an ideal tool for studies of arbuscular mycorrhizal growth response. High-throughput phenotyping is an imaging-based method that enable researchers to analyse the growth of plants non-invasively of the same plants during the their life-span, on a large scale (Berger et al. 2012; Walter et al. 2012). High throughput phenotyping methods repeatedly track the growth rate of a plant during the course of its life using imaging methods which use digital cameras with subsequent software image analysis. High throughput phenotyping has been used to study a plants' stress and/or genetic variation in many previous studies, for example in barley (Honsdorf et al. 2014; Neumann et al. 2015), in pea (Humplík et al. 2015) and in chickpea (Atieno et al. 2017). Furthermore, recent study of Watts-Williams et al. (2019c) illustrated the perspective of utilisation of HTP in exploring the growth response of plant to AM. Briefly, it was found that the growth of three different plant species in response to AM and Zn addition differed considerably over the life of the plant, and that plant biomass at the final destructive harvest did not reflect these dynamic changes over time. In summary, HTP systems provide a useful tool to analyse the complex interactions among plants, AM and environmental factors, over time.

Objectives of the thesis

While it is well established that AM have great potential to improve the growth and nutrition of plants, much work remains to be done to explore and realise these benefits, especially in agriculturally important plant species. In the current project, the effects of forming AM, and soil P and Zn fertilisation, on the growth and mineral nutrition of both plant vegetative tissue and edible portions of agriculturally important plants was explored. Specifically, the main objectives of the work presented in this thesis were to:

1. Assess the impact of arbuscular mycorrhizal fungal inoculation on the growth, individual nutritional responses and plant ionome of different agriculturally important plant species. (*Chapter two*)

2. Determine the effect of arbuscular mycorrhizal fungal inoculation on the productivity, and the concentration of phytic acid and mineral nutritional bioavailability (i.e. Zn and Fe) in the grains of agriculturally important genotypes of durum wheat. (*Chapter three and four)*

3. Assess the impact of phosphorus and/or zinc additions on the plant mycorrhizal response in the growth, yield, concentration of phytic acid and nutritional quality in durum wheat grains. (*Chapter three and four*)

4. Analyse the impact of arbuscular mycorrhizal fungal inoculation with phosphorus and zinc in soil on the plant growth over time employing the high-throughput phenotyping platform. (*Chapter five*)

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Chapter 2 - Impact of an arbuscular mycorrhizal fungus on the growth and nutrition of fifteen crop and pasture plant species

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Principal Author

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Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Name of Co-Author	Stephanie J. Watts-Williams
Contribution to the Paper	Co-designed the study Co-author on the manuscript 10 %
Signature	Date 26/03/2020

Name of Co-Author	Timothy R. Cavagnaro
Contribution to the Paper	Co-designed the study Co-author on the manuscript 10 %
Signature	Date 25th March, 2020

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Chapter 3 - Arbuscular mycorrhizal fungal inoculation and soil zinc fertilisation affect the productivity and the bioavailability of zinc and iron in durum wheat

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Contribution to the Paper	Co-designed the study, Perform lab work Author on the manuscript		
Overall percentage (%)	80 %		
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Name of Co-Author	Stephanie J. Watts-Williams		
Contribution to the Paper	Co-designed the study Co-author on the manuscript 10 %		
Signature		Date	26/03/2020

Name of Co-Author	Timothy R. Cavagnaro		
Contribution to the Paper	Co-designed the study Co-author on the manuscript 10 %		
Signature		Date	25th March, 2020

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Chapter 4 - Potential for arbuscular mycorrhizal fungi to enhance the bioavailability of zinc and iron in diverse durum wheat genotypes

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Signature	Date: 20/03/2020

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Stephanie J. Watts-Williams
Contribution to the Paper	Co-designed the study Co-author on the manuscript 10%
Signature	Date: 4/5/20
Name of Co-Author	Timothy R. Cavagnaro
Contribution to the Paper	Co-designed the study Co-author on the manuscript 5%
Signature	Date: 1st MAy, 2020.
Name of Co-Author	Jason A. Able
Contribution to the Paper	Co-designed the study Co-author on the manuscript 5%
Signature	Date: 01/05/2020

Please cut and paste additional co-author panels here as required.

Potential for arbuscular mycorrhizal fungi to enhance the bioavailability of zinc and iron in diverse durum wheat genotypes

Authors: Binh T.T. Tran [a,b], Timothy R. Cavagnaro [a], Jason A. Able [a], Stephanie J. Watts-Williams [a,c]

Affiliations:

[a] The Waite Research Institute and the School of Agriculture, Food and Wine, the University of Adelaide, Waite Campus, PMB1 Glen Osmond, SA, 5064, Australia.

[b] Faculty of Agriculture and Forestry, Taynguyen University, Buon Ma Thuot city, Daklak province, 63131, Vietnam.

[c] The Australian Research Council Centre of Excellence in Plant Energy Biology, the University of Adelaide, Waite Campus, PMB1 Glen Osmond, SA, 5064, Australia.

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Abstract

Arbuscular mycorrhizal fungi (AMF) are considered to be promising symbiotic partners of food crops, including durum wheat, in order to achieve food security in low-input agricultural systems. However, the effect of arbuscular mycorrhizas (AM) on the yield and nutritional quality, particularly the mineral elements zinc (Zn) and iron (Fe), of durum wheat grain is highly variable and dependent on many factors. One of these factors is the host plant genotype. Moreover, phytic acid (PA), the main storage form of phosphorus (P) in the grain, can act as an anti-nutritional agent that reduces the bioavailability of mineral cations, including Zn and Fe in grain for human absorption. Therefore, in order to assess the true effect of AM on plant nutritional quality, determining the bioavailability of Zn and Fe is critical. In this study, we analysed PA concentrations and the bioavailability of grain Zn and Fe of 101 geographically diverse durum wheat genotypes. In addition, we examined the impact of the AMF *Rhizophagus irregularis* inoculation on ten selected genotypes with diverse PA backgrounds to soil P addition on the yield and mineral nutrition of their grain. Results showed that there was a significant variation in PA concentration throughout the panel of durum wheat genotypes. The effect of AM was greatest in low soil P conditions, and varied among genotypes. While soil P addition greatly increased grain production, it also increased grain PA concentration in all genotypes. Furthermore, in the low soil P condition, AM increased Fe and Zn uptake, therefore, increased the bioavailability of grain Zn and Fe. The result of this study can inform breeders selecting an optimal durum wheat genotype which is superior consistently in both yield and grain quality when forming AM.

Introduction

Agriculture is under ever-increasing pressure to provide food for the world`s growing population. Cereal-based diets can lead to micronutrient malnutrition and poor human health (White and Broadley 2009). It is estimated that more than 30% of the world's population suffers from "hidden hunger" as a result of deficiency in their vitamin A, iron, zinc, copper or iodine intake (FAO 2013). This means that global food demand is increasing both in terms of quantity and of nutritional quality. Therefore, in addition to yield, the traits of grain mineral nutrition such as Zn and Fe and their bioavailability, are critical purposes of breeding programs (Cakmak, 2010).

Arbuscular mycorrhizas (AM), have the potential to help meet this increasing demand (Antunes et al. 2012; Cobb 2016; Rillig et al. 2019). Arbuscular mycorrhizas are symbiotic associations formed between arbuscular mycorrhizal fungi (AMF) and roots of many agriculturally important plant species (Baum et al. 2015; Smith and Smith 2011; Smith and Read 2008). They are known for their beneficial impacts on plants under biotic and abiotic stress, such as drought (Cavagnaro 2016), salinity (Porcel et al. 2012), pathogen pressure (Baum et al. 2015), heavy metal contamination (Sharma and Agrawal 2006) and soil nutrient deficiency (Smith and Smith 2011). Associations with AMF can lead to improvement in crop productivity and concentration of nutrients in the grain or other edible portions of plants (Lehmann and Rillig 2015; Lehmann et al. 2014; Ma et al. 2019; Pellegrino et al. 2015; Tran et al. 2019). For this reason, the use of AMF in agricultural systems has attracted great interests towards sustainable production purposes, which not only increase plant nutrition but also reduce the fertilizer and water used.

Plant identity (species and genotype) is one of the main factors causing variation in the effect of AMF on plant biomass and nutrition. The variation in plant response to arbuscular mycorrhizal fungal colonisation in diverse plant genotypes has been illustrated in many agriculturally important crops such as bread wheat (Zhu et al. 2001), durum wheat (Ellouze et al. 2016), sorghum (Watts-Williams et al. 2019a), chickpea (Bazghaleh et al. 2018) and maize (An et al. 2010). Variation in mycorrhizal colonisation among plant genotypes may be based on the differences in their root length and density (Bazghaleh et al. 2018; Kashiwagi et al. 2006), or root exudates which influence the timing and vigour of mycorrhizal colonisation (Ellouze et al. 2016; Venturi and Keel 2016). Moreover, plant genotypic diversity may also affect nutrient uptake and

consequently nutritional quality of their food parts, for example in sorghum (Cobb et al. 2016), and water relations in tomato (Bowles et al. 2016) and *Medicago truncatula* (Watts-Williams et al. 2019b). Therefore, the selection and cultivation of major crops should be based on the selection of the genotypes that are compatible with AMF in order to better harness the benefits of AM (An et al. 2010; Singh et al. 2012).

Durum wheat (*Triticum turgidum* L. ssp. *durum*) is one of the most important commercial cereal crops, and is grown globally, for example Turkey, Canada, Italy and Australia. Durum wheat flour is mainly used for pasta production and specialty breads which are consumed as staple foods across the Middle East (Abecassis et al. 2012) and other regions of the world (Kezih et al. 2014; Magallanes-López et al. 2017). Therefore, the grain nutritional quality affects human nutrition, especially the mineral nutrients which contribute to reducing hidden hunger. Even though the mycorrhizal growth responsiveness of durum wheat has been reported to be relatively low (Al-Karaki 1998; De Vita et al. 2018; Tran et al. 2019b), the benefit of AM to durum wheat could be substantial in certain conditions (Ercoli et al. 2017; Tran et al. 2019a). Modern wheat breeding programs and intensive agriculture practice have improved durum wheat productivity three-fold since 1900, and released a great number of durum wheat cultivars varying in agronomic traits (Sissons 2012). Traits from improvement efforts include grain yield and responding to fertiliser and pesticide inputs (Lehmann et al. 2012). However, the yield improvement in commercial genotypes was found to correlate with a reduction in grain Zn and Fe concentration, in comparison with wild genotypes (Vázquez et al. 2018). In addition, there has been a concern that the genotypic selection processes may lead to the reduction in the capacity of formation and function of AM in the more modern genotypes in comparison with the wild ones (Hetrick et al. 1995; Turrini et al. 2016; Zhu et al. 2001). Because the most beneficial condition for the function of AM is low nutrient soil, high fertile soil conditions such as high phosphorus (P) may inhibit mycorrhizal colonisation (Richardson et al. 2011). However, evidence for this concern is still inadequate (Lehmann et al. 2012) and may not be applicable to durum wheat (De Vita et al. 2018; Ellouze et al. 2015).

Research examining genotypic variation in durum wheat to the formation of AM are limited, and inconclusive (De Vita et al. 2018; Dupont 2019; Ellouze et al. 2016; Ellouze et al. 2018; Singh et al. 2012). Testing the effect of AMF inoculation on Canadian durum wheat genotypes, Ellouze et al. (2016), Ellouze et al. (2018) and Dupont (2019)

did not find any significant impact of plant genotype on mycorrhizal root colonization, nor AMF community composition in the soil. Conversely, Singh et al. (2012) and De Vita et al. (2018) illustrated great variation in mycorrhizal colonisation of different durum wheat genotypes.

Improving the grain nutritional quality is an essential breeding consideration which benefits global food production. Therefore, nutritional traits such as the bioavailability of micronutrients important for human health (e.g., Zn and Fe) are important to consider across a wide range of durum wheat genotypes. However, most studies assessed the concentration of these mineral nutrients (Coccina et al. 2019; Ercoli et al. 2017; Zhang et al. 2019) but did not extend the work to determine their bioavailability. The bioavailability of Zn and Fe in grain depends heavily on the concentration of phytic acid (PA) - the dominant storage form of P in seeds. Phytic acid can strongly bind positively charged proteins, amino acids and many nutritionally important minerals including Zn and Fe, forming insoluble phytate-mineral complexes. Therefore, PA is considered as the main anti-nutrient compound that may lead to human Zn and Fe malnutrition, especially in the human population that rely on a cereal-based diet (Black et al. 2013).

In this study, we present results of an experiment that builds on our recent finding that AM increased PA concentration in a single durum wheat variety, which consequently reduced the bioavailability of grain Zn and Fe (Tran et al. 2019a). Here, the concentration of PA and estimated bioavailability of Zn and Fe of 101 geographically diverse varieties of durum wheat were analysed. These results informed our choice of ten genotypes that were then used for examining the responses to AMF under low and high soil phosphorus conditions, in terms of yield and nutrition (including Zn and Fe bioavailability). We had three specific research questions:

1. How do the diverse genotypes of durum wheat vary in grain yield, nutrition and PA concentration?
2. To what extent does soil phosphorus fertilisation interact with arbuscular mycorrhizal fungal inoculation?
3. To what extent does arbuscular mycorrhizal fungal inoculation affect durum wheat nutrition and micronutrient bioavailability?

Materials and methods

A panel of 101 geographically and genetically diverse durum wheat genotypes (Table S1) was used to estimate the potential for diversity in grain PA accumulation. This set was chosen from a core population of 315 genotypes (see Liu et al. (2018), Table S1). The seeds of 100 genotypes (DBA-Aurora excluded) were harvested in 2014 from field trial conditions in Breeza, New South Wales, Australia (31.25°S, 150.46°E). DBA-Aurora seeds were sourced from two different seasons for the experiments conducted in 2017, Roseworthy, South Australia, (34.54°S, 138.69°E). All grain samples (approximately 5 g of each) were dried in an oven at 60 ^{o}C for 72 hours then ground to a fine powder to homogenise using a Retsch mill MM400 (Germany). Two weighed sub-samples of this flour were taken to analyse the concentration of PA, Zn and Fe in the grain (see below for sample analysis). The bioavailability of Zn and Fe, including the molar ratio of phytic acid to zinc (PA: Zn) and phytic acid to iron (PA: Fe), were calculated. Based on the initial results obtained from the PA concentration study (Figure 1), their commercial importance, and geographic origin, a refined sub-set of ten genotypes from a total of four countries were then selected for further experimentation (Figure 1).

Soil and plant preparation

The soil used was a 9:1 (*w/w*) mixture of sand and field soil. This field soil was collected from the grounds of the University of Adelaide's Waite Arboretum, Australia (-34.969209, 138.631397). The field soil was sieved to <2 mm to eliminate any coarse debris, autoclaved twice, and oven dried at 60 ^{o}C before being mixed thoroughly with the washed and autoclaved fine sand and is referred as 'soil' hereafter. The field soil was an Urrbrae red-brown earth (Alfisol) with 6.14 pH, 55 mg N kg^{-1} KCl-extractable ammonium, 11 mg N kg^{-1} KCl-extractable nitrate, 13.5 mg P kg^{-1} of Colwell P, 81 mg Fe kg^{-1} DTPA-extractable Fe concentration and 17 mg Zn kg^{-1} DTPA-extractable Zn. The mixing resulted in soil with 19 mg Fe kg^{-1} DTPA-extractable Fe and 2.9 mg Zn kg^{-1} DTPA-extractable Zn.

The mycorrhizal inoculum used was composed of dry soil, spores, external hyphae of *Rhizophagus irregularis* WFVAM10, and root fragments of colonised Marigold (*Tagetes patula*) plants produced in December 2018. In the mycorrhizal treatment pots, 140 g (10% pot weight) of the *R. irregularis* WFVAM10 inoculum was

mixed thoroughly with soil to a total of 1,400 g. Meanwhile, the other half of the pots were mock-inoculated whereby each pot of 1400 g soil was given 15 mL of aqueous filtrate of 20 % suspension (*w/v*) of the *R. irregularis* inoculum, filtered twice using Whatman #1 paper (Li et al. 2006), mixed well and pots filled.

To investigate the effects of soil P availability, half of the soil and inoculum mixture was amended with 20 mg P kg^{-1} in the form of KH_2PO_4 solution (10 mg P mL^{-1}) to both mycorrhizal and mock-inoculated pots to form two P treatments, with and without P addition. These treatments had Colwell P concentration of 7.8 and 25.2 mg P kg^{-1}, respectively, and are referred to as Low P and High P hereafter.

Seeds of the durum wheat genotypes were sterilised in 10 % sodium hypochlorite solution for 5 min then rinsed with running reverse osmosis (RO) water before being germinated in an incubation chamber at 25 °C in the dark. Germinated seeds were transferred to autoclaved sand and grown in a greenhouse (see below for greenhouse conditions) for 10 days. The seedlings were then transplanted to the previously prepared pots, with one plant per pot. Each treatment was replicated five times, with a total of 200 pots.

Plant growth and harvest

Plants were grown in a controlled environment greenhouse on the Waite Campus of The University of Adelaide, during the months of late July to early October 2019. Conditions in the greenhouse ranged from 10.8 to 29.1 ^{o}C temperature, 21.5 – 75.3 % relative humidity and supplemental lighting in a 16/8 day/night photoperiod. The pots were arranged randomly on the greenhouse benches and their positions were rotated once per week. Plants were watered to 10 % of the soil weight, three times weekly with RO water. Once per week, the pots were fertilised (weekly) with 10 mL of modified Long-Aston solution omitting P (Cavagnaro et al. 2010). Plants were also amended with 30 mg N per pots in the form of NH_4NO_3 solution in the second, fourth and sixth week of the growing course which resulted 90 mg N per pot in total.

All plants were destructively harvested 77 days after transplanting. The durum wheat spikelets were cut from shoots, shoots were cut at soil level and roots were separated from soil, washed and dried. Then the fresh weights of spikelets, shoot and roots of each plant were measured. Between 100 – 300 mg of fresh roots was sub-sampled and placed into a 50 % (*v/v*) ethanol solution. After drying at 60 °C for 72 hours, the dry

weights of remaining root biomass, shoot biomass and spikelet mass of each plant were measured. Grains were then separated from spikelets using threshing board and then the number of grains per plant and grain dry weight were determined. Grains were then ground to fine flour as described previously and three subsamples were taken for PA, elemental and protein analyses.

Sample analysis

Phytic acid concentrations were measured using a phytic acid/total phosphorus assay kit (Megazyme, Ireland), to measure PA concentration in durum wheat grain following the protocol of Megazyme manufacturer.

Grain elemental concentrations were determined as follow: 200 mg of finely ground grain was digested using a 2 mL of nitric acid and 0.5 mL hydrogen peroxide (Miller 1998). The plant digests were diluted with RO water and then analysed for concentrations of elements including P, Zn, and Fe by inductively-coupled plasma atomic emission spectroscopy ICP-AES (Thermo Jarrell Ash Corp., Franklin, MA, USA).

Protein concentrations in grains were determined through Dumas nitrogen (N) (Simonne et al. 1997). A weighed (150 mg) subsample of fine grain was taken to determine the grain N according to Dumas method using the rapid N exceed Elementar (Germany).

Root mycorrhizal colonisation was determined according to the gridline intersect method described by Giovannetti and Mosse (1980) as follows: fresh roots fixed in ethanol for 48 hours were rinsed with RO water and then cleared in 10 % potassium hydroxide at room temperature for seven days. Cleared roots were rinsed and then stained in 5 % ink in vinegar (modified from Vierheilig et al. (1998) at 60 °C for 10 min before being de-stained in acidified water for 12 hours, then washed and moved to 50 % glycerol solution for storage. The number of intersects of colonised root and total root of stained root samples dispersed on the gridline Petri dish were counted through microscope. The percentage of colonisation of root was then calculated.

Statistical analysis

The Low P and High P data were analysed separately due to the strong influence of soil P addition on every response variable. For each of Low P and High P data sets, two-way ANOVA were used and mycorrhiza (*Myc*) and genotype (*Genotype*) treatments as fixed factors for grain dry weights, concentration of mineral nutrients (P, Zn and Fe), protein

content, PA concentration and molar ratio of PA: Zn and PA: Fe. Where significant differences were found, Tukey's *post hoc* test was used for comparison among treatment means. Prior to undertaking data analysis, data were log-transformed where needed to meet assumptions of the ANOVA. In order to report on the effect of soil P addition, the Low P and High P data were compared by *t*-test for each response variable. All statistical analyses were performed using IBM SPSS Statistics 25.

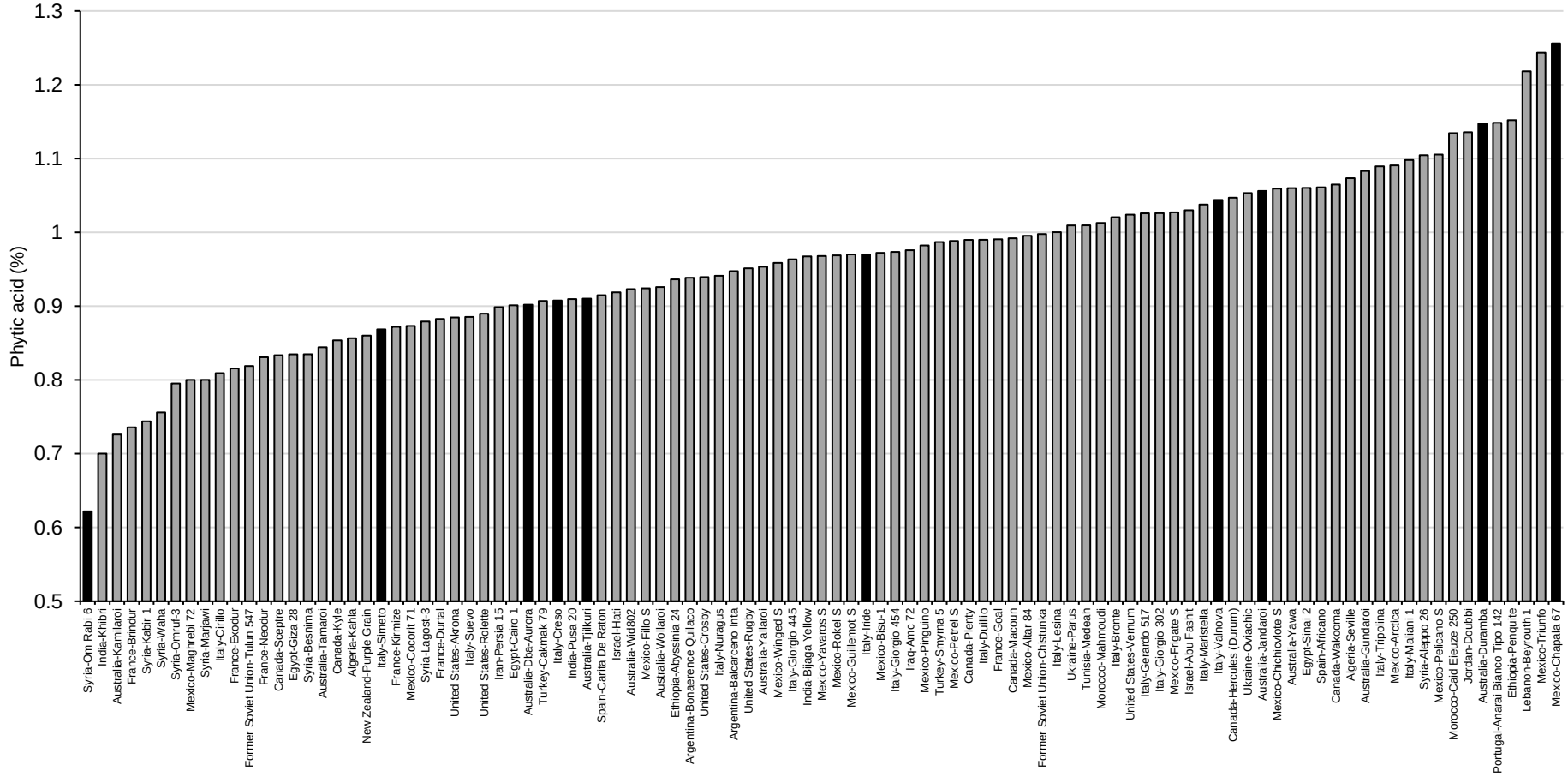
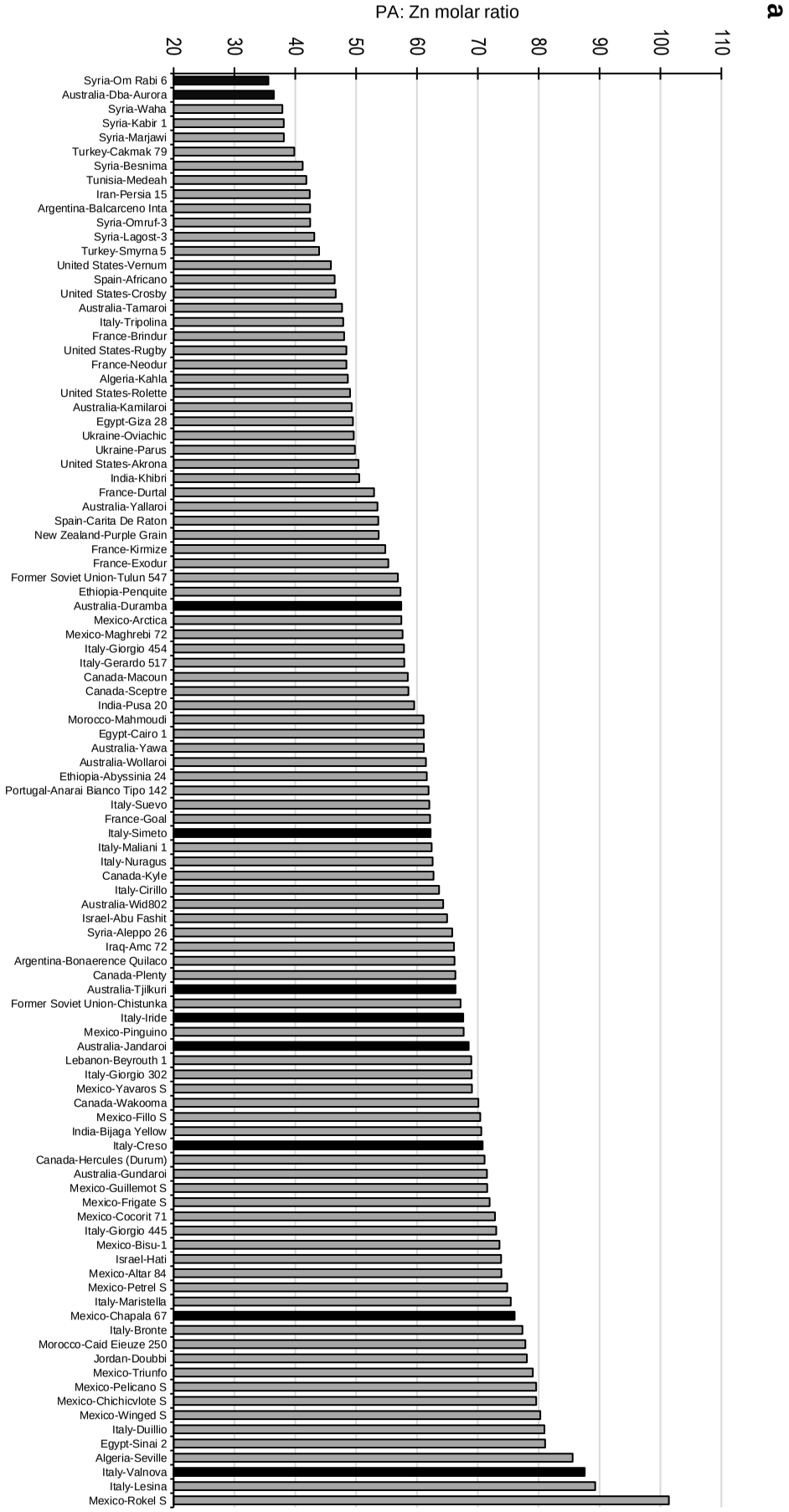


Figure 1. Phytic acid concentration (%) of 101 different varieties of durum wheat, the ten selected genotypes for further study are in black.


a
PA: Zn molar ratio
20
30
40
50
60
70
80
90
100
110
Syria-Om Rabi 6
Australia-Dba-Aurora
Syria-Waha
Syria-Kabir 1
Syria-Marjawi
Turkey-Cakmak 79
Syria-Besnima
Tunisia-Medeah
Iran-Persia 15
Argentina-Balcarceno Inta
Syria-Omruf-3
Syria-Lagost-3
Turkey-Smyrna 5
United States-Vernum
Spain-Africano
United States-Crosby
Australia-Tamaroi
Italy-Tripolina
France-Brindur
United States-Rugby
France-Neodur
Algeria-Kahla
United States-Rolette
Australia-Kamilaroi
Egypt-Giza 28
Ukraine-Oviachic
Ukraine-Parus
United States-Akrona
India-Khibri
France-Durtal
Australia-Yallaroi
Spain-Carita De Raton
New Zealand-Purple Grain
France-Kirmize
France-Exodur
Former Soviet Union-Tulun 547
Ethiopia-Penquite
Australia-Duramba
Mexico-Arctica
Mexico-Maghrebi 72
Italy-Giorgio 454
Italy-Gerardo 517
Canada-Macoun
Canada-Sceptre
India-Pusa 20
Morocco-Mahmoudi
Egypt-Cairo 1
Australia-Yawa
Australia-Wollaroi
Ethiopia-Abyssinia 24
Portugal-Anarai Bianco Tipo 142
Italy-Suevo
France-Goal
Italy-Simeto
Italy-Maliani 1
Italy-Nuragus
Canada-Kyle
Italy-Cirillo
Australia-Wid802
Israel-Abu Fashit
Syria-Aleppo 26
Iraq-Amc 72
Argentina-Bonaerence Quilaco
Canada-Plenty
Australia-Tjilkuri
Former Soviet Union-Chistunka
Italy-Iride
Mexico-Pinguino
Australia-Jandaroi
Lebanon-Beyrouth 1
Italy-Giorgio 302
Mexico-Yavaros S
Canada-Wakooma
Mexico-Fillo S
India-Bijaga Yellow
Italy-Creso
Canada-Hercules (Durum)
Australia-Gundaroi
Mexico-Guillemot S
Mexico-Frigate S
Mexico-Cocorit 71
Italy-Giorgio 445
Mexico-Bisu-1
Israel-Hati
Mexico-Altar 84
Mexico-Petrel S
Italy-Maristella
Mexico-Chapala 67
Italy-Bronte
Morocco-Caid Eieuze 250
Jordan-Doubbi
Mexico-Triunfo
Mexico-Pelicano S
Mexico-Chichicvlote S
Mexico-Winged S
Italy-Duillio
Egypt-Sinai 2
Algeria-Seville
Italy-Valnova
Italy-Lesina
Mexico-Rokel S

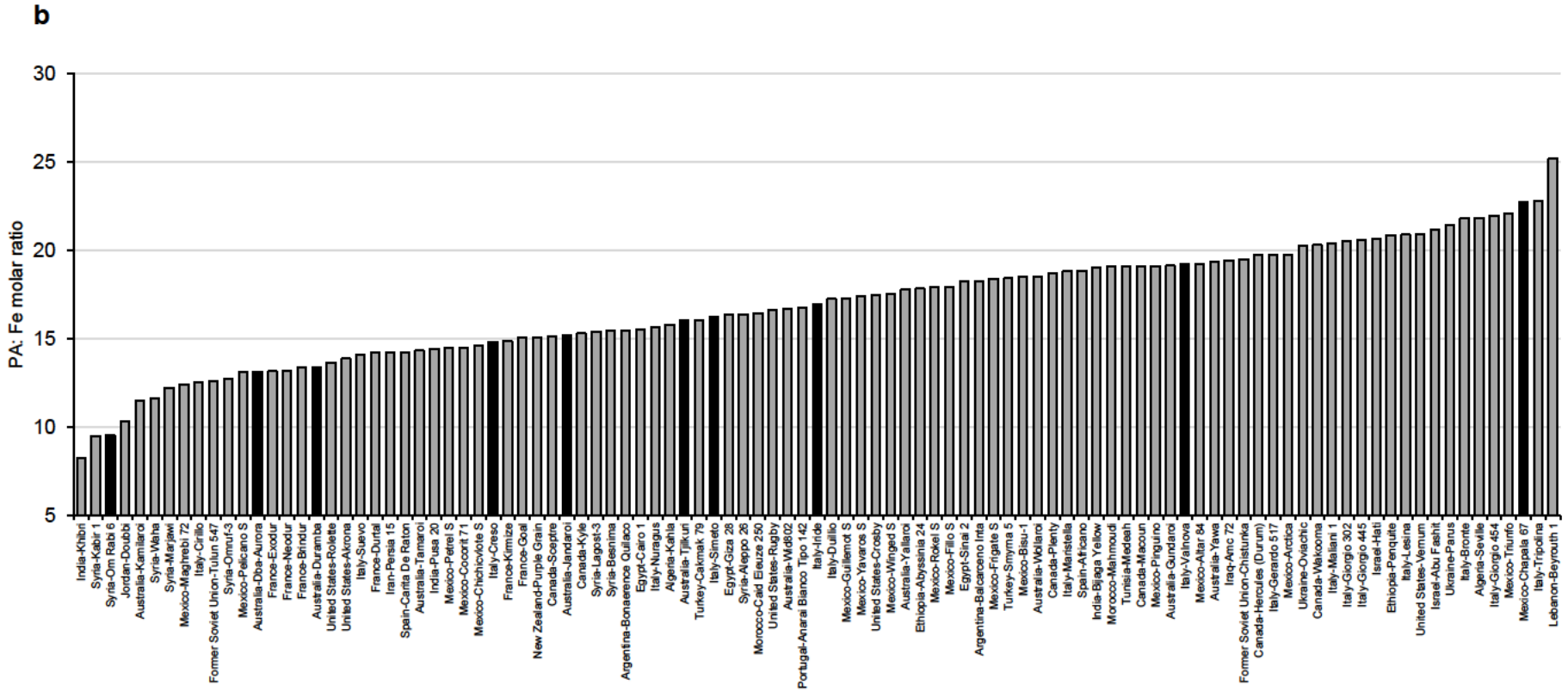


Figure 2. Molar ratio of phytic acid (PA) to zinc (Zn) concentration and ion (Fe) concentration of 101 different varieties of durum wheat. The ten selected varieties for further study are in black.

Results

Preliminary experiment

In the first part of our experiment (preliminary experiment, hereafter), the concentration of PA and estimated bioavailability of Zn and Fe of 101 geographically diverse varieties of durum wheat were analysed. This informed our choice of ten genotypes that were used in the main experiment to examine the responses to arbuscular mycorrhizal fungus under low and high soil phosphorus conditions, in terms of grain weight per plant and nutrition (including Zn and Fe bioavailability.

The bioavailability of Zn and Fe in grain from 101 durum wheat genotypes

Nutritional traits including PA, Zn and Fe concentrations were measured on 101 genetically diverse durum wheat genotypes grown under standardised field conditions and ground to a fine powder (flour). Phytic acid concentrations in the diverse genotypes ranged from 0.62 % to 1.26 % (Figure 1). There was also a weak correlation (data not shown) of $R^2 = 0.28$ between total grain P and PA concentration found across 101 analysed durum wheat genotypes.

In order to estimate the bioavailability of Zn and Fe, the molar ratio of PA: mineral commonly has been used (Ma et al. 2019; Weaver and Kannan 2002). In the case of Zn, a PA:Zn molar ratio higher than 15, between 5 and 15 and lower than 5 refers to low, medium and high bioavailability, respectively (Gibson 2006). While with Fe, a PA:Fe molar ratio higher than 1 associates with low bioavailability (Hurrell and Egli 2010). The molar ratio of PA: Zn ranged from 35.6 – 101.4 and the molar ratio of PA: Fe ranged from 8.3 – 25.2 (Figure 2).

Main experiment

In order to examine the responses of ten chosen durum wheat genotypes to arbuscular mycorrhizal fungus under low and high soil phosphorus conditions, the grain dry weight, grain protein content, grain P concentration, grain Zn concentration, grain Fe concentration, grain PA concentration were measured and the estimated bioavailability of Zn and Fe were calculated.

The effect of soil P availability on response variables

Generally, increased addition of soil P had strong effect compared to low P in all parameters analysed. In particular, the addition of P fertiliser to the soil increased yield

and concentrations of protein, P and PA in the grain, but it also led to decreased concentrations of grain Zn and Fe (Table 1). The combination of increased PA alongside decreased Zn and Fe in the grain meant that PA: Zn and PA: Fe ratios increased markedly in the High P treatment, by three and five times, respectively. Thus, the estimated bioavailability of Zn and Fe in High P grain was heavily decreased compared to in the Low P grain.

Some of the most interesting findings from the rest of the low P and high P data sets were grain dry weight, grain Zn and Fe concentrations, grain phytate and protein concentrations and grain Zn and Fe bioavailability.

Grain dry weight

In the Low P treatment, analysis of grain DW data revealed a significant two-way interaction between *Myc* and *Genotype* (Table 2, Figure 3a) but a significant main effect of *Genotype* on the grain DW at High P (Table 2, Figure 3b). At Low P level, in both mock-inoculated plants and mycorrhizal plants, Iride (Italy) had the highest grain DW (2.37 ± 0.14 g and 1.89 ± 0.14 g for mock-inoculated and mycorrhizal plants, respectively) and Chapala 67 (Mexico) produced the lowest grain weight (0.74 ± 0.14 g and 0.64 ± 0.14 g for mock-inoculated and mycorrhizal plants, respectively). When mock-inoculated, Iride had more grain than Valnova (Italy; 1.10 ± 0.15 g) and Tjilkuri (Australia; 1.51 ± 0.22 g) genotypes, whereas their mycorrhizal plants showed no difference (Figure 3a). At High P, Iride also had the highest grain DW (3.49 ± 0.17 g) and was greater than four other genotypes including: Om Rabi 6 (Syria; 2.64 ± 0.17 g), Duramba (Australia; 2.20 ± 0.17 g), Valnova (2.15 ± 0.17 g), and Chapala 67 (Mexico; 1.57 ± 0.17 g) (Figure 3b).

Grain PA and protein concentrations

In terms of grain PA concentration, there was a statistically significant main effect of *Genotype* in both Low P (Table 2 and Figure 4a) and High P (Table 2 and Figure 4b) treatments. At Low P, Chapala 67 had the highest concentration of PA in grain (0.931 ± 0.057 %) and was greater than eight other genotypes excluding Jandaroi (Figure 4a). At High P, the grain PA concentration of Duramba (1.45 ± 0.054 %) genotype was the highest, and was greater than four others genotypes including: Iride (1.11 ± 0.05 %), Om Rabi 6 (1.16 ± 0.05), Creso (1.20 ± 0.05), and Dba-Aurora (1.20 ± 0.05 %) (Figure 4b).

In terms of grain protein content, there was a significant interaction between *Myc*Genotype* at Low P (Table 2, Figure 5a) and a statistically significant main effect of *Myc* and of *Genotype* was found in High P (Table 2, Figure 5b). At Low P, while all genotypes showed no difference in grain protein content when inoculated with the AMF *R. irregularis* , among mock-inoculated plants, Iride had the highest grain protein content (0.42 ± 0.02 g plant^{-1}) and was greater than three other genotypes including: Duramba (0.27 ± 0.03 g plant^{-1}), Valnova (0.26 ± 0.03 g plant^{-1}) and Jandaroi (Australia; 0.22 ± 0.03 g plant^{-1}) (Figure 5a). At High P, when the *Myc* main effect was considered, mycorrhizal plants (i.e. pooled over genotypes) had lower protein content than mock-inoculated plants with mean values of 0.39 ± 0.01 g plant^{-1} and 0.42 ± 0.01 g plant^{-1}, respectively. In terms of the *Genotype* main effect, Iride had the highest protein content (0.47 ± 0.01 g plant^{-1}), and was greater than two other genotypes including: Valnova (0.37 ± 0.01 g plant^{-1}) and Chapala 67 (0.31 ± 0.01 g plant^{-1}) (Figure 5b).

Grain Zn and Fe concentrations

For the grain Zn concentration, there was a statistically significant main effect of *Myc* and of *Genotype* on at Low P (Table 2, Figure 6a) but only *Genotype* main effect at High P (Table 2 and Figure 6b). At Low P, when the main effect of *Myc* was considered, the pooled mean over genotype of mycorrhizal plants had higher Zn concentration in grain than mock-inoculated plants with 129.02 ± 3.57 mg kg^{-1} and 104.95 ± 3.57 mg kg^{-1}, respectively. In terms of the *Genotype* main effect, Chapala 67 had the highest Zn concentration in grain (209.4 ± 7.98 mg kg^{-1}) and was greater than all nine other genotypes. Om Rabi 6 and Iride had the lowest Zn concentration in grain (86.51 ± 7.98 and 88.79 ± 7.98 mg kg^{-1}, respectively) and were lower than three other genotypes including: Jandaroi (132.63 ± 7.98 mg kg^{-1}), Valnova (129.40 ± 7.98 mg kg^{-1}) and Duramba (125.75 ± 7.98 mg kg^{-1}) (Figure 6a). At High P, Chapala 67 had the highest Zn concentration in grain (150.7 ± 5.89 mg kg^{-1}, which was greater than all others genotypes. Zn concentration in grain of Iride was the lowest (61.7 ± 5.89 mg kg^{-1}) and was lower than three other genotypes including: Duramba (116.04 ± 5.89 mg kg^{-1}), Valnova (108.81 ± 5.89 mg kg^{-1}) and Jandaroi (96.43 ± 5.89 mg kg^{-1}) (Figure 6b).

In terms of grain Fe concentration, analysis of data revealed statistically significant main effects of both *Myc* and *Genotype* at Low P (Table 2 and Figure 7a) but only a main effect of *Genotype* at High P treatments (Table 2 and Figure 7b). At Low P, when the main effect of *Myc* was considered, the Fe concentration pooled means of

mycorrhizal plants was higher than mock-inoculated plants with 83.01 ± 3.39 mg kg^{-1} and 61.17 ± 3.39 mg kg^{-1}, respectively. In terms of the *Genotype* main effect, Tjilkuri had the highest Fe concentration in grain (91.85 ± 7.59 mg kg^{-1}) and was greater than Iride (55.87 ± 7.59 mg kg^{-1}) and Om Rabi 6 (51.71 ± 7.59 mg kg^{-1}) (Figure 7a). At High P, Chapala 67 (69.68 ± 3.41 mg kg^{-1}) had the highest Fe concentration in grain, and was greater than six other genotypes including: Tjilkuri (52.79 ± 3.41 mg kg^{-1}), Simeto (52.03 ± 3.41 mg kg^{-1}), Creso (50.82 ± 3.41 mg kg^{-1}), Dba-Aurora (48.29 ± 3.41 mg kg^{-1}), Om Rabi 6 (46.53 ± 3.41 mg kg^{-1}) and Iride (44.1 ± 3.41 mg kg^{-1}) (Figure 7b).

Grain Zn and Fe bioavailabilities

The molar ratios of PA to mineral elements (Zn/Fe) were used to represent the estimated bioavailabilities of Zn and Fe in grain.

In the case of the molar ratio of PA: Zn in grain, there were statistically significant main effects of both *Myc* and *Genotype* at Low P (Table 2, Figure 8a) but only a significant main effect of *Genotype* at High P (Table 2 and Figure 8b). At Low P, when the main effect of *Myc* was considered, the pooled mean over genotype of PA: Zn molar ratio in grain in mycorrhizal plants was lower than in mock-inoculated plants, with 4.68 ± 0.15 and 5.28 ± 0.14, respectively. In terms of the *Genotype* main effect, Iride had the highest PA: Zn molar ratio in grain (5.93 ± 0.32) and was greater than Tjilkuri (4.33 ± 0.31) and Dba-Aurora (4.26 ± 0.32) (Figure 8a). At High P, Iride also had the highest PA: Zn molar ratio in grain (18.18 ± 0.81) which was greater than four other genotypes including: Duramba (12.12 ± 0.81), Simeto (14.29 ± 0.81), Valnova (12.68 ± 0.81), and Chapala 67 (9.20 ± 0.81) (Figure 8b).

Similarly, for the molar ratio of PA: Fe in grain, there were statistically significant main effects of both *Myc* and *Genotype* at Low P (Table 2 and Figure 9a) whereas a significant main effect of *Genotype* was found at High P (Table 2 and Figure 9b). At Low P, when the main effect of *Myc* was considered, the mycorrhizal plants (pooled over genotype) had lower PA: Fe molar ratios in grain than the mock-inoculated plants (pooled over genotype) with PA: Fe molar ratio pooled mean values of 6.55 ± 0.25 and 7.51 ± 0.24, respectively. For the *Genotype* main effect, Chapala 67 had the highest PA: Fe molar ratio in grain (9.58 ± 0.56) while and was greater than five other genotypes including: Om Rabi 6 (7.11 ± 0.56), Duramba (6.56 ± 0.56), Valnova (6.36 ± 0.56), Dba-Aurora (5.22 ± 0.56) and Tjilkuri (4.93 ± 0.56) (Figure 9a). At High P, Om Rabi 6 and

Tjilkuri genotypes had the highest PA: Fe molar ratio in grain (21.24 ± 0.94 and 21.34 ± 0.94, respectively), which was greater than in Chapala 67 (16.68 ± 0.94) genotype (Figure 9b).

Table 1. T-test summary table of *p*-values comparing Low P and High P for all responses variables. Significant probabilities ($p \leq 0.05$) are highlighted with boldface.

Variables	Grain dry weight (g)	Zn (mg kg^{-1} grain)	Fe (mg kg^{-1} grain)	PA (%)	Molar ratio PA: Zn	Molar ratio PA: Fe	Protein content
p	**≤ 0.0005**	**≤ 0.0005**	**≤ 0.0005**	**≤ 0.0005**	**≤ 0.0005**	**≤ 0.0005**	**≤ 0.0005**

Table 2. ANOVA summary table of *p*-values without and with phosphorus amendment (Low P and High P) for all responses variables. One-way ANOVA for mycorrhizal colonisation (factor is *Genotype*); two-way ANOVA for all others variables (factors are *Myc* and *Genotype*). Significant probabilities ($p \leq 0.05$) are highlighted with boldface.

Variables	Low P		
	Myc	*Genotype*	*Myc * Genotype*
Mycorrhizal colonisation (%)	-	0.218	-
Grain dry weight (g)	0.031	≤ 0.0005	**0.004**
Protein content (g)	0.007	≤ 0.0005	**0.016**
Zn (mg kg^{-1} grain)	**≤ 0.0005**	**≤ 0.0005**	0.11
Fe (mg kg^{-1} grain)	**≤ 0.0005**	**0.003**	0.403
PA (%)	0.118	**≤ 0.0005**	0.436
Molar ratio PA: Zn	**0.005**	**0.002**	0.305
Molar ratio PA: Fe	**0.006**	**≤ 0.0005**	0.845

Variables	High P		
	Myc	*Genotype*	*Myc * Genotype*
Mycorrhizal colonisation (%)	-	**≤ 0.0005**	-
Grain dry weight (g)	0.382	**≤ 0.0005**	0.678
Protein content (g)	**0.014**	**≤ 0.0005**	0.19
Zn (mg kg^{-1} grain)	0.153	**≤ 0.0005**	0.692
Fe (mg kg^{-1} grain)	0.659	**≤ 0.0005**	0.996
PA (%)	0.95	**0.001**	0.962
Molar ratio PA: Zn	0.152	**≤ 0.0005**	0.506
Molar ratio PA: Fe	0.92	**0.01**	0.4

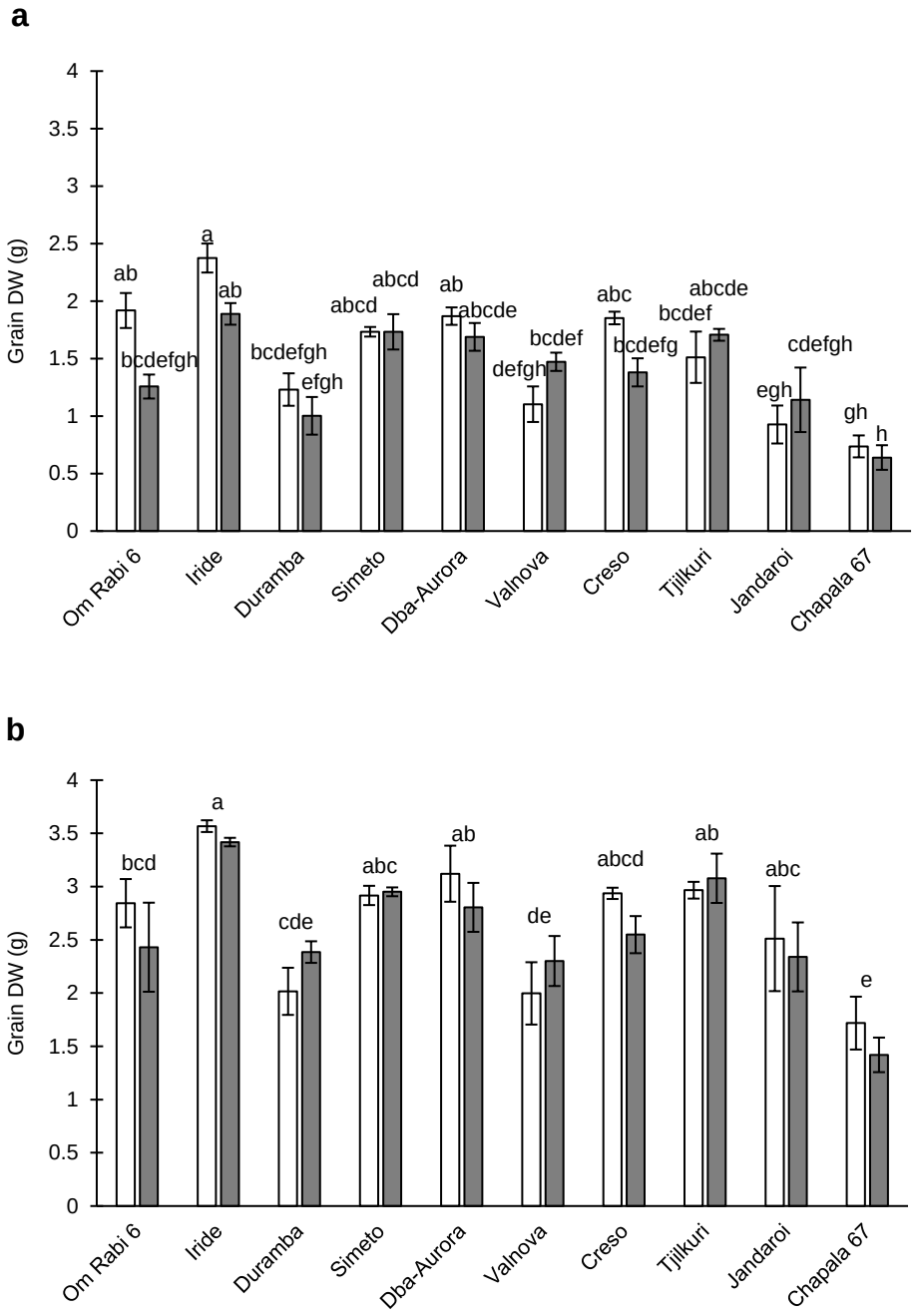


Figure 3. The dry weight (DW) at harvest of grain at Low P (a) and High P (b) of ten durum wheat varieties and either inoculated with the arbuscular mycorrhizal fungus *Rhizophagus irregularis* (grey bars) or mock-inoculated (white bars). Values are mean ± s.e., $n = 5$. Means for grain DW at Low P followed by the same letter (a, b, c) do not differ significantly by Tukey`s *post hoc* test at $p < 0.05$. Means for grain DW at High P of each variety (pooled mean) followed by the same letter do not differ significantly by Tukey`s *post hoc* test at $p < 0.05$. Different set of variables are non-comparable. See Table 2 for ANOVA results.

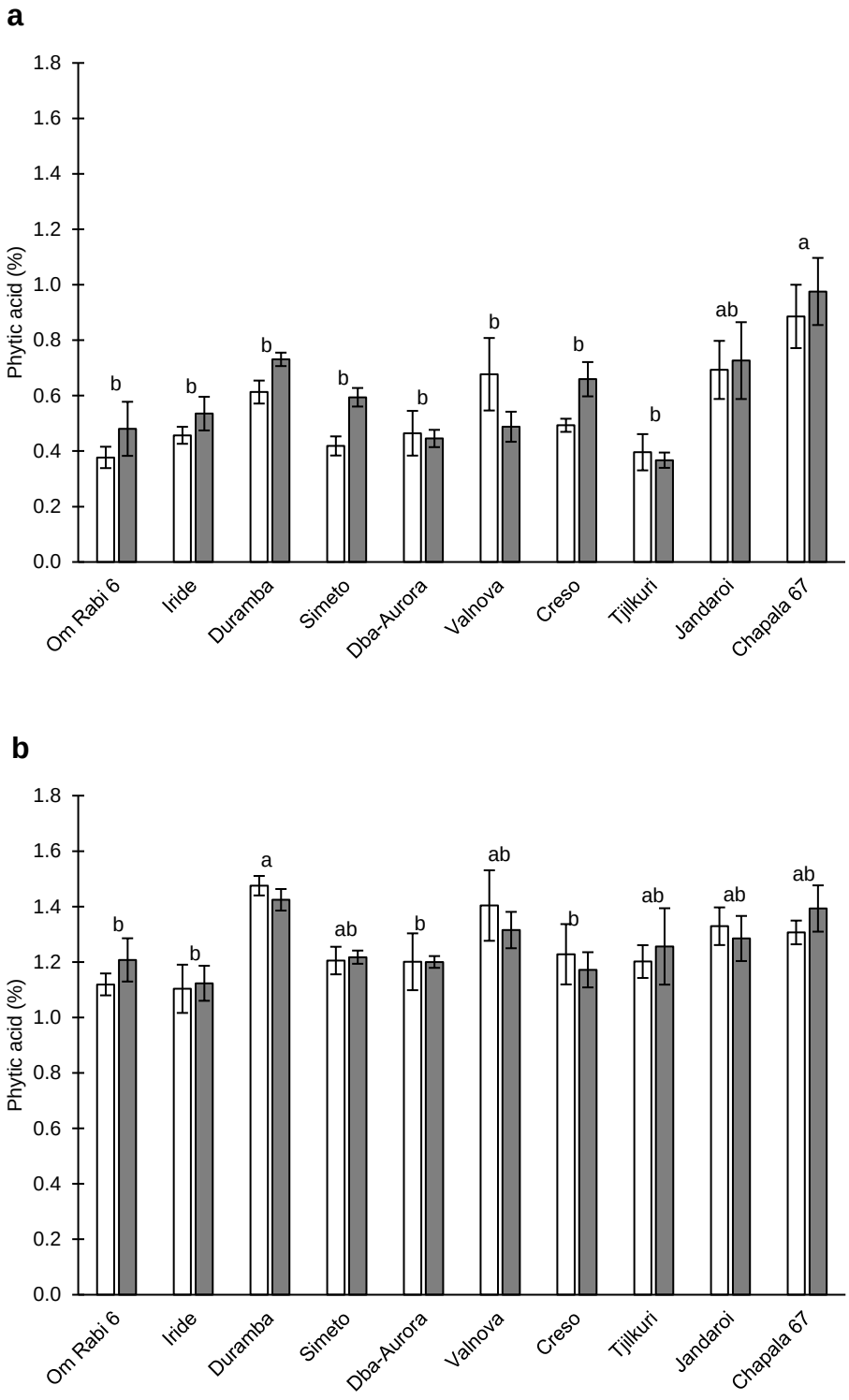


Figure 4. Phytic acid concentrations in grain of ten durum wheat varieties at Low P (a) and High P (b), and either inoculated with the arbuscular mycorrhizal fungus *R. irregularis* (grey bars) or mock-inoculated (white bars). Values are mean ± s.e., $n = 5$. Means of each variety (pooled mean) at both Low P and High P followed by the same letter do not differ significantly by Tukey`s *post hoc* test at $p < 0.05$. Different set of variables are non-comparable. See Table 2 for ANOVA results

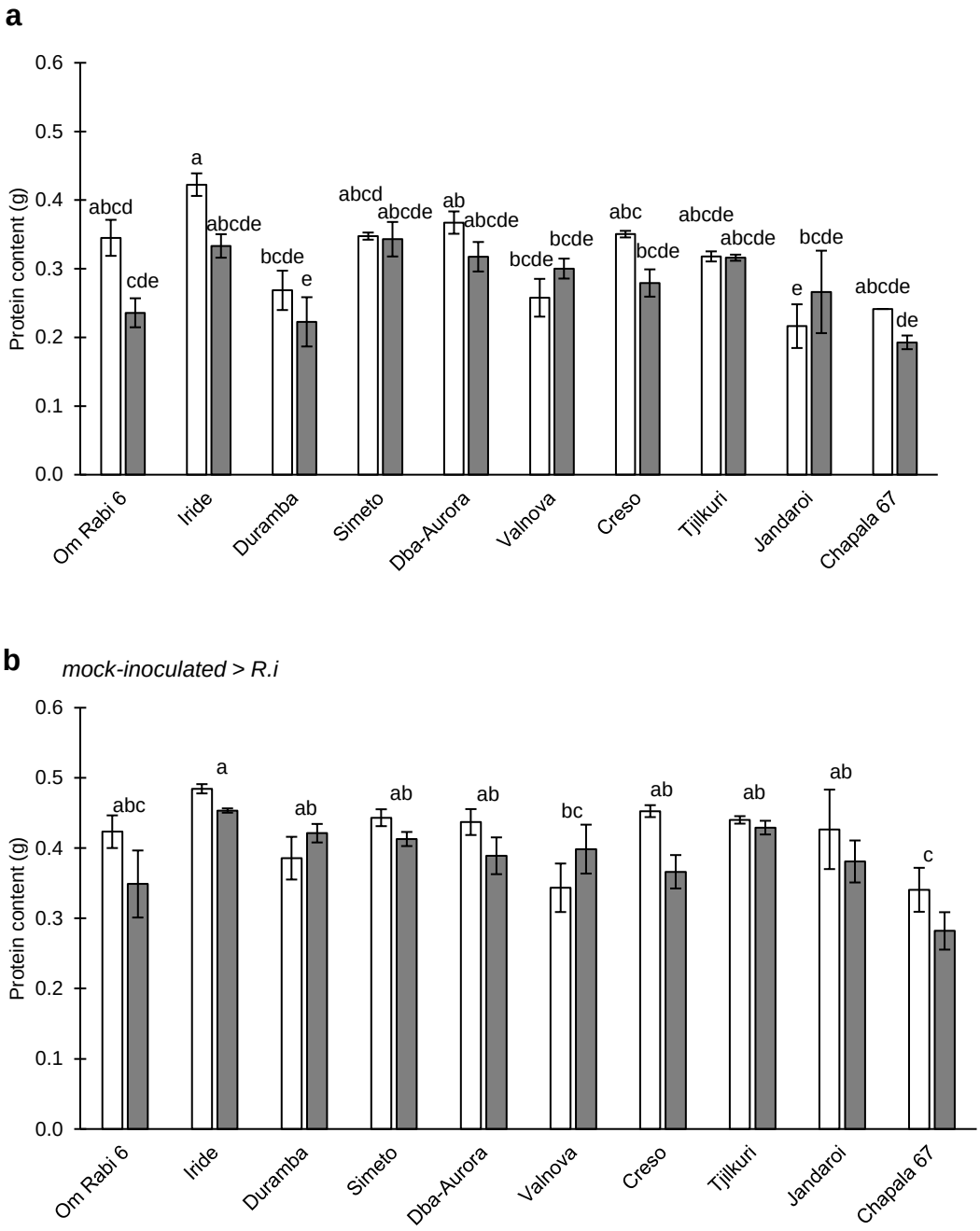


Figure 5. The protein content in grain of ten durum wheat varieties at Low P (a) and High P (b), and either inoculated with the arbuscular mycorrhizal fungus *R. irregularis* (grey bars) or mock-inoculated (white bars). Values are mean ± s.e., $n = 5$. Means for protein content at Low P followed by the same letter (a, b, c) do not differ significantly by Tukey's *post hoc* test at $p < 0.05$. Means for protein content at High P of each variety (pooled mean) followed by the same letter do not differ significantly by Tukey`s *post hoc* test at $p < 0.05$. Different set of variables are non-comparable. See Table 2 for ANOVA results.

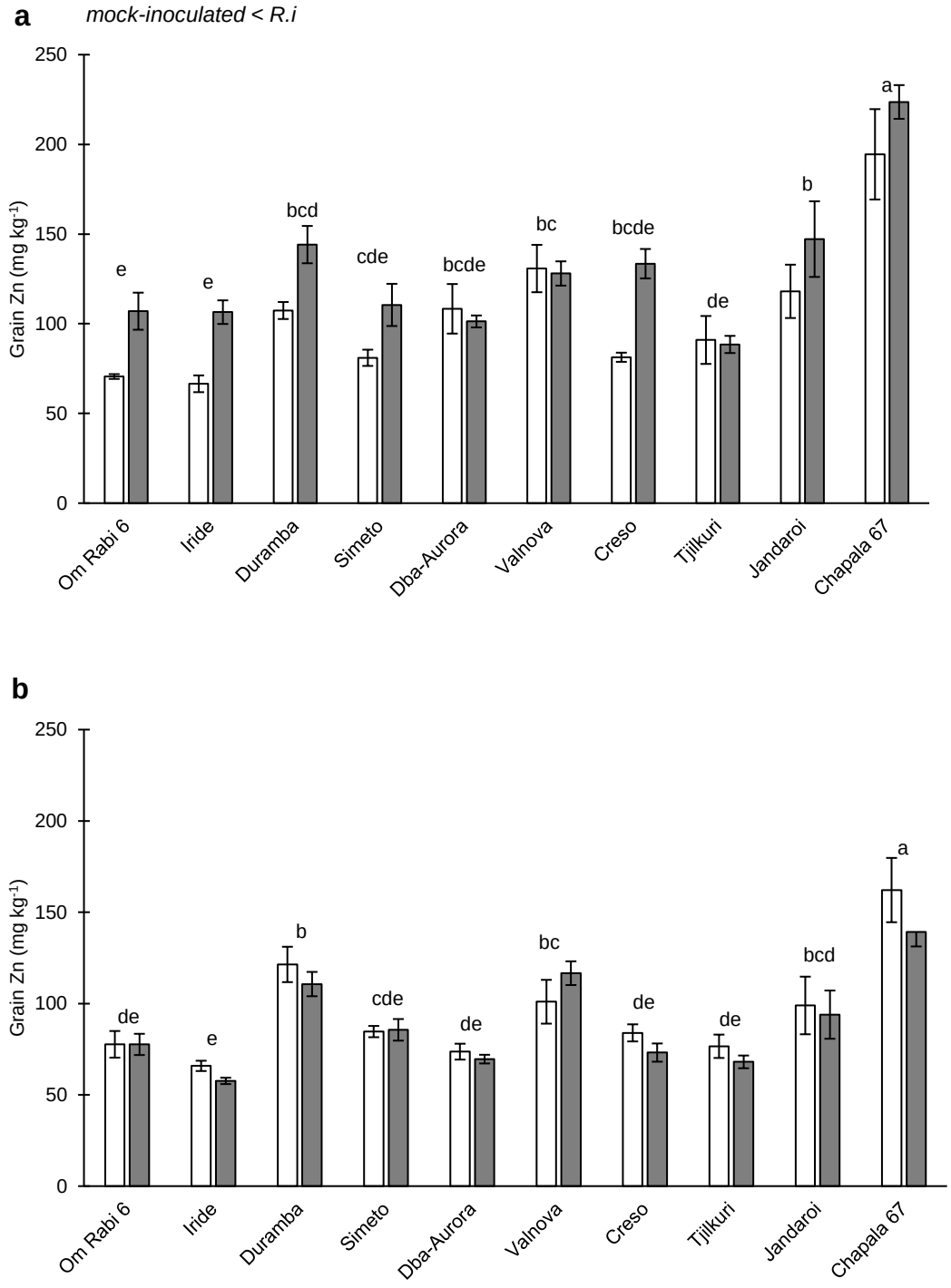


Figure 6. The concentration of zinc (Zn) in grain at Low P (a) and High P (b) of ten durum wheat varieties and either inoculated with the arbuscular mycorrhizal fungus *R. irregularis* (grey bars) or mock-inoculated (white bars). Values are mean ± s.e., $n = 5$. Means of each variety (pooled mean) at both Low P and High P followed by the same letter do not differ significantly by Tukey`s *post hoc* test at $p < 0.05$. Different set of variables are non-comparable. See Table 2 for ANOVA results.

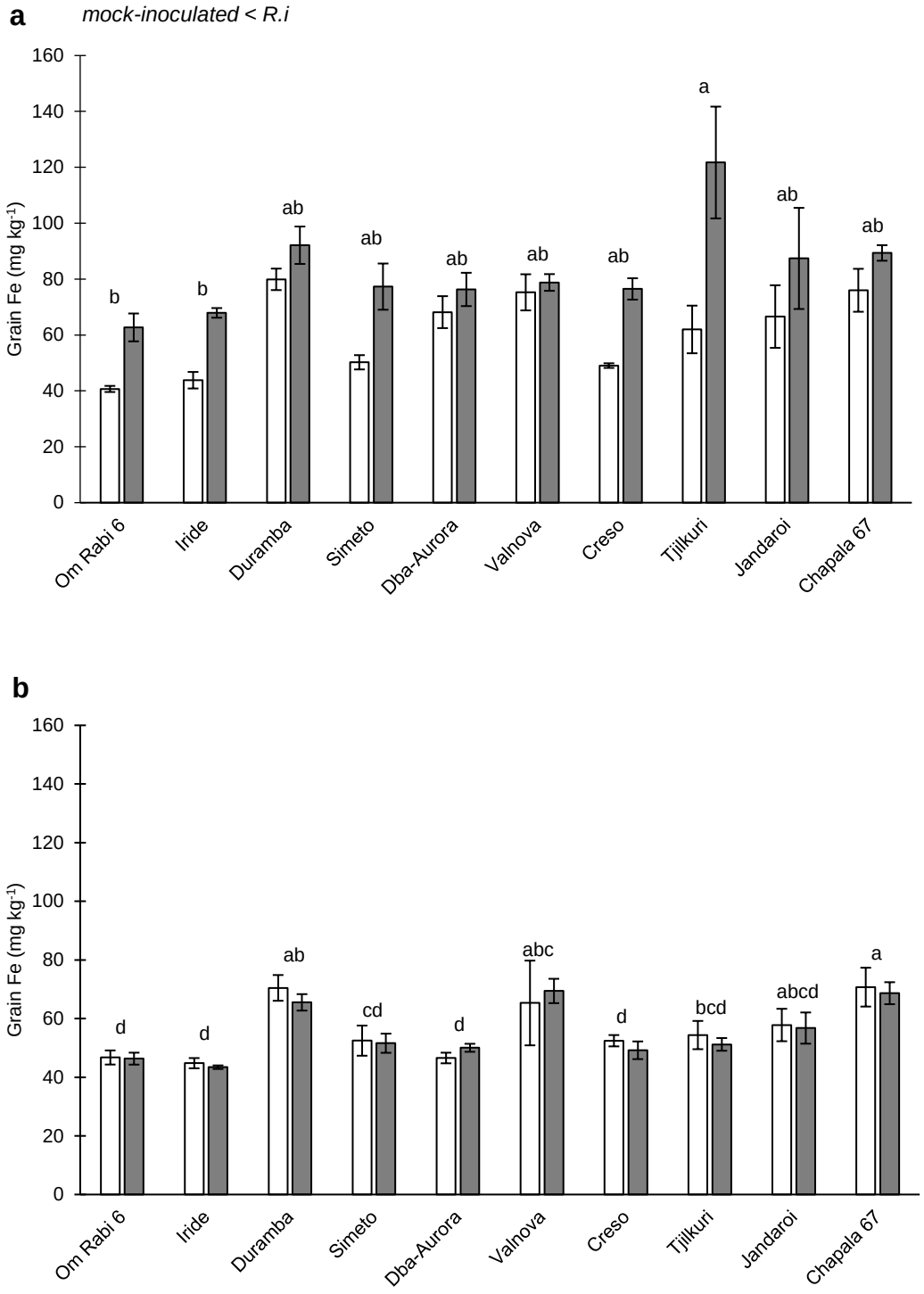


Figure 7. The concentration of iron (Fe) in grain at Low P (a) and High P (b) of ten durum wheat varieties and either inoculated with the arbuscular mycorrhizal fungus *R. irregularis* (grey bars) and mock-inoculated (white bars). Values are mean ± s.e., $n = 5$. Means of each variety (pooled mean) at both Low P and High P followed by the same letter do not differ significantly by Tukey`s *post hoc* test at $p < 0.05$. Different set of variables are non-comparable. See Table 2 for ANOVA results.

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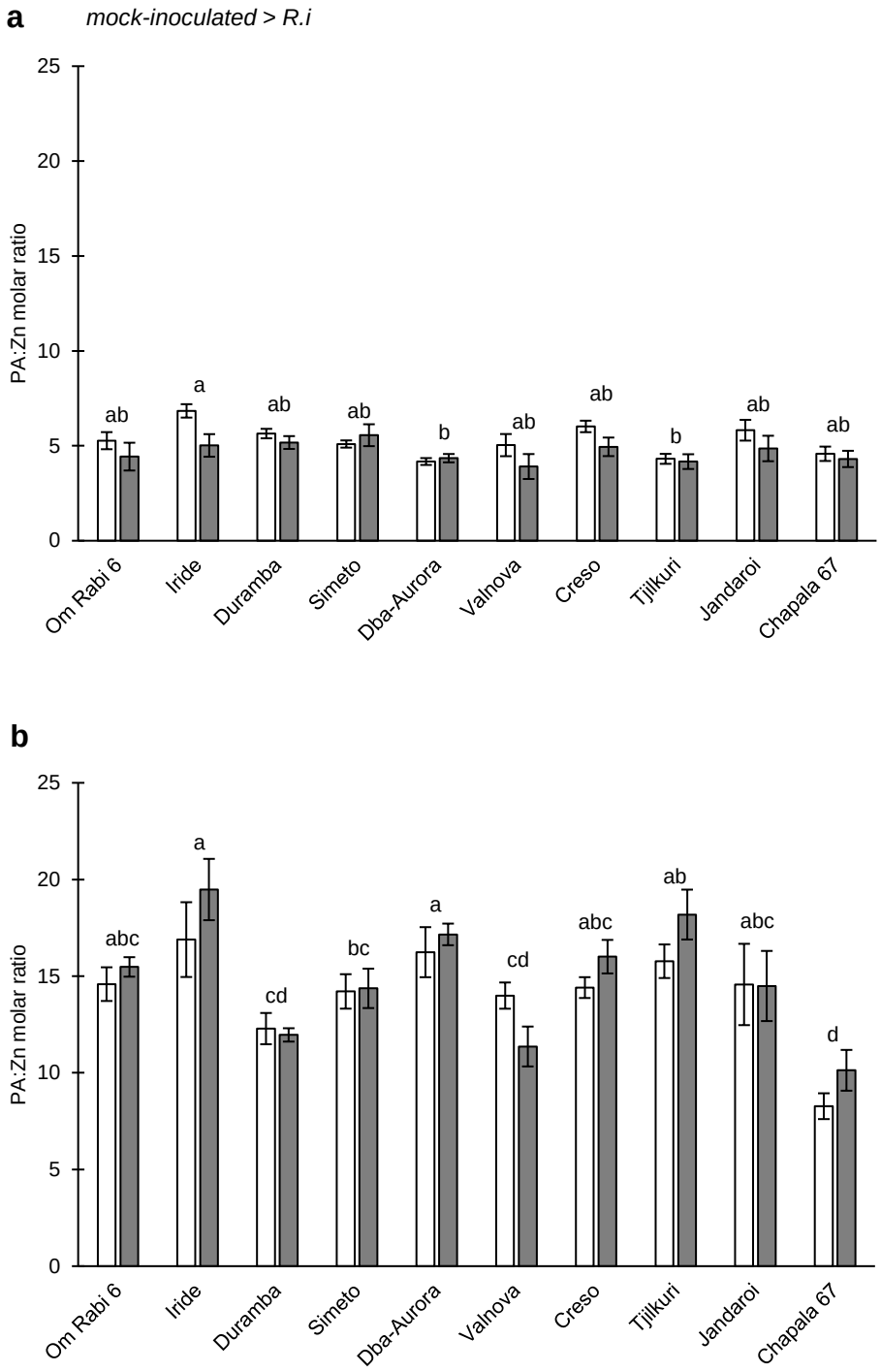

Figure 8. Molar ratios of phytic acid to Zn of ten durum wheat varieties at Low P (a) and High P (b), and either inoculated with the arbuscular mycorrhizal fungus *R. irregularis* (grey bars) or mock-inoculated (white bars). Values are mean ± s.e., $n = 5$. Means of each variety (pooled mean) at both Low P and High P followed by the same letter do not differ significantly by Tukey`s *post hoc* test at $p < 0.05$. Different set of variables are non-comparable. See Table 2 for ANOVA results.

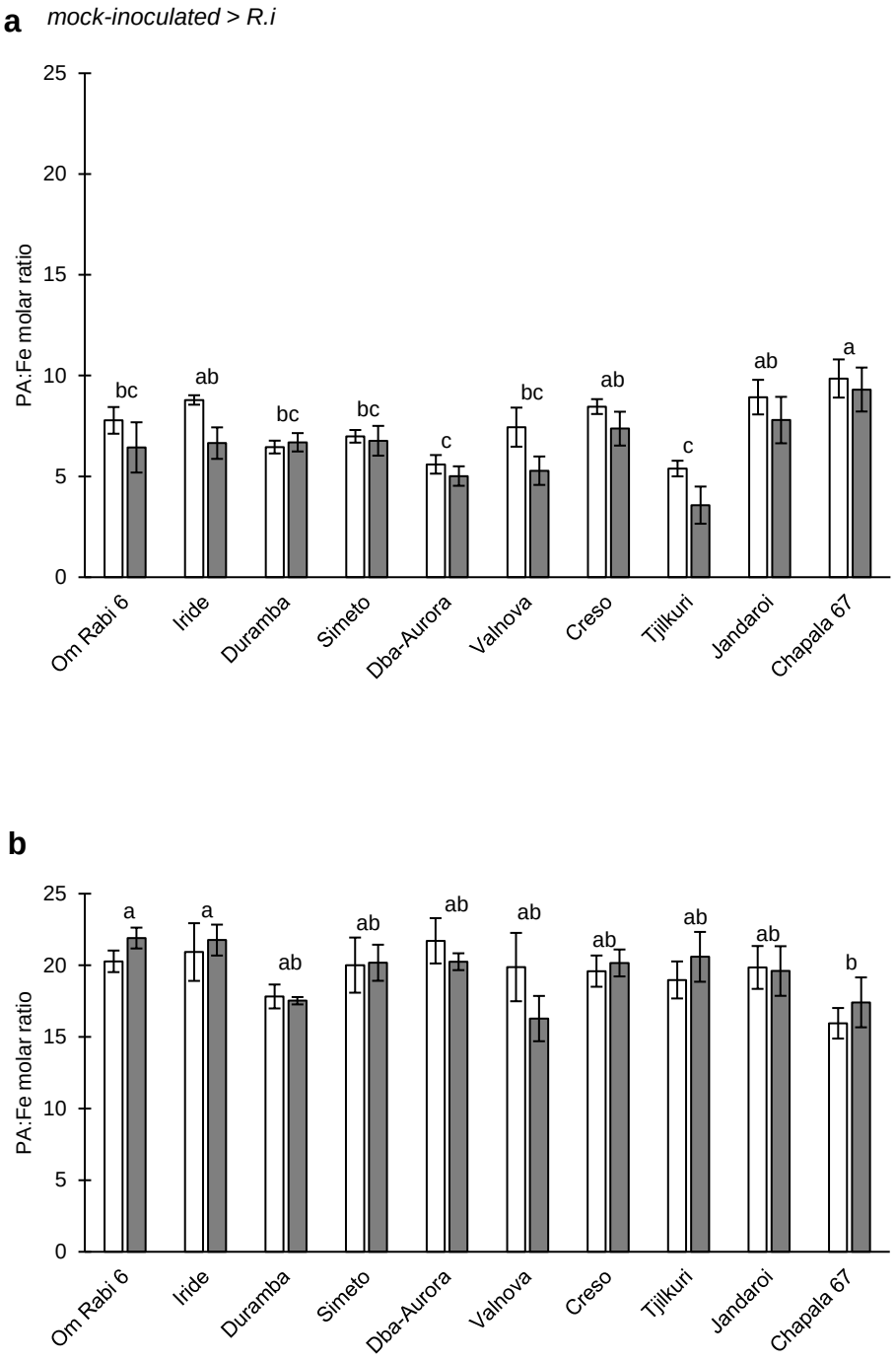

Figure 9. Molar ratios of phytic acid to Fe in grain of ten durum wheat varieties at Low P (a) and High P (b), in either inoculated with the arbuscular mycorrhizal fungus *R. irregularis* (grey bars) or mock-inoculated (white bars). Values are mean ± s.e., $n = 5$. Means of each variety (pooled mean) at both Low P and High P followed by the same letter do not differ significantly by Tukey`s *post hoc* test at $p < 0.05$. Different set of variables are non-comparable. See Table 2 for ANOVA results.

Discussion

This study focused on the effect of soil P, AMF inoculation and plant genotype on the productivity and nutrition of durum wheat grain. Results are now discussed in agronomic and human nutritional contexts.

Mycorrhizal inoculation affects durum wheat nutrition and bioavailability in low P soil

Arbuscular mycorrhizal fungal inoculation influenced grain nutritional quality, particularly the concentration of PA, Zn and Fe in grain when plants were grown under low soil P conditions. This was consistent with earlier studies in durum wheat and many other plant species (Lehmann and Rillig 2015; Lehmann et al. 2014). The formation of AM can result in the enhanced uptake, and ultimately grain concentration, of P, Zn and Fe in plants (Coccina et al. 2019; Ercoli et al. 2017; Goicoechea et al. 2016). This was also evident in the present study where the grain concentrations of P, Zn, and Fe were significantly increased by arbuscular mycorrhizal fungal inoculation. While increased uptake of Zn and Fe is important, it is not necessarily a good indication of the bioavailability of those nutrients in grain. Accordingly, we focused on the concentration of PA – the dominant storage form of P in seeds – and assessed the bioavailability of Zn and Fe in durum wheat grain.

Arbuscular mycorrhizas increased PA concentration in the Italian genotypes Simeto and Creso, while no effect was found in the other genotypes. The DBA-Aurora genotype in the current study did not show any difference in grain PA concentration by AM while it was significant in our previous study (Tran et al. 2019a), even though the same arbuscular mycorrhizal fungus species, *R. irregularis* was used. The differences between the two experiments are likely due to the fact that seeds in two experiments came from different sources; seeds of this experiment were obtained from a drought stress study (Liu et al. 2018) while the seeds of previous experiment originated from a non-stressed condition. The increases in Zn and Fe concentrations in the mycorrhizal plants were much stronger than the increases of PA concentration, therefore the molar ratios of PA: Zn and PA: Fe were lower in mycorrhizal plants here. This led to the increase of the estimated bioavailabilities of these two micronutrients. This positive effect of AM on the bioavailability of Zn and Fe was also found previously in maize grain (Subramanian et al. 2013). However, Subramanian et al. (2013) found that AMF

inoculation not only increased Zn and Fe concentration but also decreased PA in maize grain. This suggests that the impact of AM on PA in grain was complex and variable. In addition to plant species and genotype effects, AM effects on PA may also be influenced by soil edaphic factors (e.g. P, Zn).

Diverse durum wheat genotypes differed in PA concentration and the bioavailability of Zn and Fe

Grain PA concentration is an important food quality parameter in durum wheat, this is especially the case with wholemeal flour becoming more popular in human diets (Alan et al. 2012). Wholemeal flour is not only rich in fiber, vitamins, phenolic compounds but also PA (Liu et al. 2018). Here, the results of wholemeal flour PA concentration and the molar ratio of PA: Zn and PA: Fe was discussed.

Across the 101 durum wheat genotypes analysed here, the concentration of PA in the grain had about two-fold variation. This variation is similar to the finding of Magallanes-López et al. (2017) (0.46 - 0.95 %) in a set of 46 geographically diverse durum wheat genotypes, but the PA concentrations in the present study were higher. In contrast, our PA findings had higher variation but less PA (%) than the results of Branković et al. (2015) (1.46 – 1.68 %), who analysed PA in a set of 15 durum wheat genotypes. However, the contribution of different environmental conditions to this variation is also considerable as has been reported in many earlier studies (Al-Karaki 1998; Ercoli et al. 2017). Moreover, the range of variation in the molar ratio of both PA: Zn and PA: Fe (35.6 – 101.4 and 8.3 – 25.2, respectively), was higher than the range of PA variation. The molar ratio of PA: Fe in our data was in agreement with data of Magallanes-López et al. (2017) and Younas et al. (2019) (12.1 – 29.6 and 5.5 - 24.1, respectively). For the PA: Zn molar ratio, our data is much higher than data of Magallanes-López et al. (2017) (16.9 – 23.6) but in agreement with Erdal et al. (2002) (49 – 116). However, in this and the other studies cited herein, all durum wheat genotypes fall in the low bioavailability group for both Zn and Fe, according to the standard of WHO (Gibson 2006) and Hurrell and Egli (2010), respectively. According to Board (2005), the daily intake requirement of Zn is 8 mg day^{-1} and 11 mg day^{-1} for a female and male adults, respectively, while the daily intake requirement of Fe is 18 mg day^{-1} and 8 mg day^{-1} for a female and male adults, respectively. To meet the daily nutrient demand a female and male adult who mainly consumed food from durum wheat grain, needs to consume about 0.6 kg day^{-1} and 0.9 kg day^{-1} of flour for adequate Zn; and 2.1 kg day^{-1}

and 0.96 kg day^{-1} of flour for adequate Fe, respectively (assuming that 15% of intake Zn, Fe from grain durum wheat available for absorption). This helps to explain the widespread human malnutrition of Zn and Fe in individuals who mainly consume a monotonous diet with a cereal grain staple. Moreover, it suggests that it is important to consider two aspects of this matter together: improving both Fe and Zn concentrations and reducing PA concentrations in durum wheat genotypes. However, this is complex and remains unsolved.

Plant genotypic diversity is a considerable driver of the mycorrhizal responsiveness

It is well established that mycorrhizal responsiveness is highly dependent on plant genotype (Watts-Williams et al. 2019b), and that durum wheat is a low mycorrhizal growth responsive plant species (Al-Karaki 1998; Goicoechea et al. 2016; Tran et al. 2019b). Here we found that plant genotype significantly affected plant responses to arbuscular mycorrhizal fungal inoculation in most of the measures such as: mycorrhizal colonisation, productivity and nutritional quality of durum wheat. This was in agreement with previous studies (De Vita et al. 2018; Ercoli et al. 2017; Singh et al. 2012). In terms of mycorrhizal colonisation, genetic variation was not detected at Low P condition but genetic control was significant in the High P treatment. Mycorrhizal colonisation in the Italian genotypes (Iride, Simeto and Valnova) was less suppressed at High P than some of the Australian ones (Duramba and Jandaroi). However, high colonisation does not necessarily correlate with benefit received from the mycorrhizal symbiosis (Smith et al. 2004), and this situation was true in our data. Here, neutral and negative effects were observed in many response variables and genotypes. This suggests that the plants allocated relatively more photosynthates to the formation and maintenance of AM than the benefits that AM returned (Li et al. 2008). Here, in the Low P condition, the durum wheat grain dry weight and grain protein content were affected by the interaction between plant genotype and AM. In these two traits, variations were not only found among genotypes but also in their responses to AM within each plant genotype. Particularly, AM reduced the grain DW in highly productive genotypes such as Iride and Om Rabi 6, but increased grain DW in lower productive genotypes (Valnova and Tjilkuri) while neutral effects were found in the other genotypes. This suggests that AM can help the less productive genotypes achieve the same yield to the most productive genotype. However, in this study, we found that across yield and nutritional quality measurements, no single durum wheat genotype that formed AM had consistent, superior nor bad

performance. However, among ten genotypes, Tjilkuri and DBA-Aurora had the lowest PA: Zn and PA: Fe ratios but modest grain yield in low P condition which can be used widely across Australian soils. Therefore, in the view of finding a variety that is highly compatible and may benefit from AMF in both quantity and quality, more effort should be spent on breeding programmes. And the genotypes that positively responded to mycorrhizal colonisation can be used as potential genetic materials. These two Australian genotypes can be good candidates, especially in the context of an Australian P-deficient soil

Soil phosphorus fertilisation strongly affected mycorrhizal responses

It has been well-established that soil P availability has a strong effect on plant mycorrhizal responsiveness parameters as mycorrhizal colonisation of roots, biomass and the nutrient uptake pathway (Chiu and Paszkowski 2019). In this study, the ten durum wheat genotypes differed considerably in their performance due to soil P addition. It is not surprising that the increase of P in soil significantly increased the plant productivity, grain protein content, grain P and PA concentrations. Moreover, the effect of soil P addition on those parameters was much stronger in comparison with the effect of AM. In particular, in the High P addition treatment, the formation of AM did not have a significant impact on grain yield, and even reduced grain protein content and grain P accumulation. In terms of PA concentration, our findings were consistent with the result of Ma et al. (2019) and Ryan et al. (2008) in bread wheat that increased PA concentration in grain was likely due to increasing P in soil. Because PA is the dominant storage form of P in seeds, therefore the increase in PA was accompanied by an increase in grain P when P was added to the soil. On the other hand, P fertilisation resulted in the decrease of Zn and Fe concentrations in grain because of the dilution effect when grain DW increased. Consequently, High P increased the grain molar ratio of PA: Zn and PA: Fe thus decreased the bioavailability of Zn and Fe. Durum wheat responded greatly in yield to P fertiliser because most modern genotypes were bred for optimal performance under high-fertility soil conditions by genotypic selection processes (Lehmann et al. 2012). This is a trade-off between high yield and low mineral nutrient bioavailability.

However, it is important to note that this study just analysed the responsiveness of durum wheat genotypes to *R. irregularis* inoculation. Therefore, arbuscular mycorrhizal responses may differ from other AMF species and/or in different conditions. For example, Al-Karaki (1998) reported an increase in biomass and yield in two durum

wheat genotypes when inoculated with *Glomus mosseae*. Further work is needed to confirm genotypes and/or AM fungal species and soil conditions which are consistently higher in AM responsiveness, greater bioavailability of Zn and Fe for selection of more ideal materials for food security and sustainable agriculture purposes.

Conclusions

The responses of ten durum wheat genotypes to inoculation with the arbuscular mycorrhizal fungus *R. irregularis*, were highly dependent on soil P availability. Overall, the impact of AM on plant yield and nutrition varied among genotypes and was most effective at Low P. In the Low P condition, *R. irregularis* inoculation increased both the concentration and bioavailability of grain Zn and Fe, which has potential implications for biofortification strategies in durum wheat. While AM increased grain P, no overall effect of AM on grain PA was found, but AM still increased PA concentration in grain of Simeto and Creso. In this study, it was evident that AM may increase the grain PA concentration, but is highly dependent on the plant genotype. Moreover, genotype diversity is significant to both grain yield and nutrient quality of durum wheat. Therefore, this result can inform the breeders seeking to select for a durum wheat genotype that can associate with AMF for greater yield and better grain Zn and Fe bioavailability for human nutrition. Further testing in field conditions will provide practical insights into the agronomic relevance of this study.

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Supplementary material

Table S1. Set of 101 durum wheat genotypes using for phytic acid and elements (P, Zn and Fe) analysis

Nº	**Origin**	**Genotype**
1	Algeria	Seville
2	Algeria	Kahla
3	Argentina	Balcarceno Inta
4	Argentina	Bonaerence Quilaco
5	Australia	Duramba
6	Australia	Yawa
7	Australia	Tamaroi
8	Australia	Jandaroi
9	Australia	Wollaroi
10	Australia	Yallaroi
11	Australia	Gundaroi
12	Australia	Wid802
13	Australia	Tjilkuri
14	Australia	Kamilaroi
15	Australia	Dba-Aurora
16	Canada	Hercules (Durum)
17	Canada	Wakooma
18	Canada	Macoun
19	Canada	Plenty
20	Canada	Kyle
21	Canada	Sceptre
22	Egypt	Sinai 2
23	Egypt	Giza 28
24	Egypt	Cairo 1
25	Ethiopia	Abyssinia 24
26	Ethiopia	Penquite

Nº	**Origin**	**Genotype**
27	Former Soviet Union	Chistunka
28	Former Soviet Union	Tulun 547
29	France	Durtal
30	France	Kirmize
31	France	Exodur
32	France	Brindur
33	France	Neodur
34	France	Goal
35	India	Pusa 20
36	India	Khibri
37	India	Bijaga Yellow
38	Iran	Persia 15
39	Iraq	Amc 72
40	Israel	Hati
41	Israel	Abu Fashit
42	Italy	Maliani 1
43	Italy	Giorgio 445
44	Italy	Gerardo 517
45	Italy	Tripolina
46	Italy	Giorgio 454
47	Italy	Maristella
48	Italy	Suevo
49	Italy	Bronte
50	Italy	Giorgio 302
51	Italy	Nuragus
52	Italy	Simeto
53	Italy	Cirillo
54	Italy	Lesina

Nº	**Origin**	**Genotype**
55	Italy	Valnova
56	Italy	Iride
57	Italy	Duillio
58	Italy	Creso
59	Jordan	Doubbi
60	Lebanon	Beyrouth 1
61	Mexico	Arctica
62	Mexico	Triunfo
63	Mexico	Chapala 67
64	Mexico	Pinguino
65	Mexico	Frigate S
66	Mexico	Guillemot S
67	Mexico	Pelicano S
68	Mexico	Maghrebi 72
69	Mexico	Winged S
70	Mexico	Altar 84
71	Mexico	Petrel S
72	Mexico	Chichicvlote S
73	Mexico	Yavaros S
74	Mexico	Bisu-1
75	Mexico	Cocorit 71
76	Mexico	Rokel S
77	Mexico	Fillo S
78	Morocco	Mahmoudi
79	Morocco	Caid Eieuze 250
80	New Zealand	Purple Grain
81	Portugal	Anarai Bianco Tipo 142
82	Spain	Africano

Nº	**Origin**	**Genotype**
83	Spain	Carita De Raton
84	Syria	Aleppo 26
85	Syria	Marjawi
86	Syria	Kabir 1
87	Syria	Waha
88	Syria	Besnima
89	Syria	Om Rabi 6
90	Syria	Lagost-3
91	Syria	Omruf-3
92	Tunisia	Medeah
93	Turkey	Smyrna 5
94	Turkey	Cakmak 79
95	Ukraine	Oviachic
96	Ukraine	Parus
97	United States	Vernum
98	United States	Rolette
99	United States	Rugby
100	United States	Akrona
101	United States	Crosby

Chapter 5 - High-throughput phenotyping reveals growth of *Medicago truncatula* is positively affected by arbuscular mycorrhizal fungi even at high soil phosphorus availability

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Principal Author

Name of Principal Author (Candidate)	Binh Thi Thanh Tran		
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Signature		Date	01/05/2020

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By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Bettina Berger		
Contribution to the Paper	Co-designed the study Critical review of manuscript 5%		
Signature		Date	04/04/2020
Name of Co-Author	Nathaniel Jewell		
Contribution to the Paper	Data analysis Co-author of manuscript 5%		
Signature		Date	04/05/2020

Name of Co-Author	Chris Brien
Contribution to the Paper	Data analysis Co-author of manuscript 5%
Signature	Date 1/5/2020

Name of Co-Author	Stephanie J. Watts-Williams
Contribution to the Paper	Co-designed the study Co-author on the manuscript 40 %
Signature	Date 4/5/20

Name of Co-Author	Timothy R. Cavagnaro
Contribution to the Paper	Co-designed the study Critical review of manuscript 5%
Signature	Date 1st May, 2020.

Please cut and paste additional co-author panels here as required.

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RESEARCH ARTICLE

High-throughput phenotyping reveals growth of *Medicago truncatula* is positively affected by arbuscular mycorrhizal fungi even at high soil phosphorus availability

Binh T. T. Tran[1,2] | **Timothy R. Cavagnaro**[1] | **Nathaniel Jewell**[1,3] | **Chris Brien**[1,3] | **Bettina Berger**[1,3] | **Stephanie J. Watts-Williams**[1,4]

[1]The Waite Research Institute and the School of Agriculture, Food and Wine, The University of Adelaide, SA, Australia

[2]Faculty of Agriculture and Forestry, Taynguyen University, Buon Ma Thuot City, Daklak province, Vietnam

[3]Australian Plant Phenomics Facility, The Plant Accelerator, The University of Adelaide, Glen Osmond, SA, Australia

[4]The Australian Research Council Centre of Excellence in Plant Energy Biology, The University of Adelaide, Glen Osmond, SA, Australia

Correspondence
Stephanie J. Watts-Williams, The Waite Research Institute and the School of Agriculture, Food and Wine, The University of Adelaide, SA, Australia.
Email: stephanie.watts-williams@adelaide.edu.au

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Societal Impact Statement

Arbuscular mycorrhizal fungi (AMF) may contribute to enhanced yield and nutrition of host plants for the purpose of sustainable agriculture. However, the growth response of the host plant to mycorrhizal colonization is generally only measured at harvest, and thus management decisions regarding AMF are made using only a single time point. This study highlights that AMF can provide growth benefits to the host plant over its life. Greater knowledge of how plants respond to AMF over time will improve understanding of how the association functions and ultimately lead to improved management decisions regarding AMF in an agricultural context.

Summary

- Colonization by arbuscular mycorrhizal fungi (AMF) can result in variable responses in the growth and mineral nutrition of host plants, and is highly dependent on soil nutrient condition; limited studies have addressed the effects of AMF on plant growth over time. The aim of this study was to investigate the AMF effects on plant growth over the life of the plant, and interactions with soil phosphorus (P) and zinc (Zn) availability.
- We used a high-throughput shoot phenotyping system to examine the temporal growth responses to AMF and soil P and Zn availabilities in the pasture legume *Medicago truncatula*. Plants were either inoculated with *Rhizophagus irregularis* or mock-inoculated, and were examined under two soil P and five soil Zn availability treatments. Plants were then destructively harvested to obtain final biomass and shoot nutrition data.
- The growth of *M. truncatula* plants over time responded very differently to AMF depending on the soil P availability. At low P, projected shoot area and absolute growth rate (AGR) became increasingly greater in the mycorrhizal plants over the course of the experiment. At high P, there was a positive growth response to AMF until approximately 40 days after planting, after which the AGR of the

BTTT and SJWW contributed equally to this work.

non-mycorrhizal plants increased and the response to AMF became neutral. Zinc availability was highly interactive with P availability, but not with AMF inoculation.
- This research demonstrates that growth responses to mycorrhizal fungi change over the plant's life, and are highly dependent on soil P availability.

KEYWORDS
arbuscular mycorrhizal fungi, high-throughput phenotyping, *Medicago truncatula*, mycorrhizal growth response, phosphorus, temporal response, zinc

1 | INTRODUCTION

Arbuscular mycorrhizas, the symbiotic associations between arbuscular mycorrhizal fungi (AMF) in the soil and the roots of the majority of terrestrial plants, have been considered for their potential to enhance both the biomass and nutrient quality of plants, especially under soil nutrient deficiency conditions (Baum, El-Tohamy, & Gruda, 2015; Bowles, Jackson, Loeher, & Cavagnaro, 2017; Rillig et al., 2019; Zhang, Lehmann, Zheng, You, & Rillig, 2019). Recently, AMF inoculum has been produced commercially as a "biofertilizer" for field use, with the aim to reduce the use of chemical fertilizer without a loss in yield; however, the merits of such biofertilizers are still under debate (Hart, Antunes, Chaudhary, & Abbott, 2018; Rillig et al., 2019; Ryan & Graham, 2018). The effect of AMF on plant biomass at harvest can be highly variable, and range from highly positive, to neutral, through to negative (Kim, Eo, Lee, Park, & Eom, 2017; Tran, Watts-Williams, & Cavagnaro, 2019; Watts-Williams, Emmett, et al., 2019). The main drivers of plant growth responses to AMF are host plant identity (species and genotype), AMF identity (species, isolate, and diversity), and environmental (soil nutrition and moisture) factors (Facelli et al., 2014; Klironomos, 2003). However, the interactions between AMF and these factors are complex; deeper insights are required into the effect of interactions between factors on mycorrhizal growth responses.

Two soil elements that have demonstrated interactions with arbuscular mycorrhizas are phosphorus (P) and zinc (Zn) (Bolan, 1991; Cavagnaro, 2008). Plant acquisition of P and Zn is highly interactive; plants can be affected by "P-induced Zn deficiency" through the dilution of Zn and other micronutrients in tissues (Robson & Pitman, 1983). This is particularly observed when the soil is high in plant-available P (through fertilizer application or naturally) and low in plant-available Zn, and can lead to decreased concentrations of Zn in the plant (Broadley, White, Hammond, Zelko, & Lux, 2007). Given that AMF can acquire both P and Zn for uptake into plant tissues, nutrient interactions are even more complex in a mycorrhizal plant; previous studies into AMF, Zn, and P interactions demonstrated that soil P availability needs to be low to see a real improvement in plant Zn nutrition by AMF (Nguyen, Cavagnaro, & Watts-Williams, 2019; Watts-Williams & Cavagnaro, 2012). The mycorrhizal growth response is large in low soil P conditions as plants depend on the P mycorrhizal pathway (Facelli et al., 2014). When P availability in the soil is high, mycorrhizal colonization of plant roots is generally suppressed. Moreover, the growth responses to AMF may become neutral or negative, although the P mycorrhizal pathway can still be actively contributing to plant P uptake (Grace, Cotsaftis, Tester, Smith, & Smith, 2009; Smith, Smith, & Jakobsen, 2004).

Meanwhile, the impact of soil Zn both in deficient and toxic concentrations can result in positive mycorrhizal growth responses, due to the dual effects of AMF on plant Zn nutrient uptake (Watts-Williams, Tyerman, & Cavagnaro, 2017). In Zn-deficient soil, AMF can increase Zn uptake via the mycorrhizal pathway of uptake, with external hyphae acquiring soil Zn and transferring it to the plant as for P (Lehmann, Veresoglou, Leifheit, & Rillig, 2014). In toxic soil Zn conditions, AMF can protect plants from excessive accumulation of Zn (Chen, Li, Tao, Christie, & Wong, 2003; Christie, Li, & Chen, 2004); however, this protective mechanism is not well understood. The availability of P and Zn in soil is highly interactive (Epstein & Bloom, 2005); for example, high soil P availability may reduce the plant availability of Zn (Ryan, McInerney, Record, & Angus, 2008), and therefore affect the Zn nutrition of the plants (Zhang et al., 2012). Mycorrhizal plants have the additional interaction of AMF-mediated P and Zn uptake (Watts-Williams, Patti, & Cavagnaro, 2013); specifically, AMF improvements in plant P nutrition and thence biomass can lead to "P-induced Zn deficiency" in plant tissues (Zhu, Christie, & Laidlaw, 2001). Therefore, it is important to study the effect of AMF on plant growth and nutrition under a range of soil P and Zn availabilities.

An example of a plant species that responds positively to AMF inoculation in terms of growth is *Medicago truncatula*, a pasture legume and model plant species commonly used in fundamental studies of mycorrhizal functioning due to the wealth of molecular tools available (Garmier, Gentzbittel, Wen, Mysore, & Ratet, 2017; Harrison, Dewbre, & Liu, 2002; Tadege et al., 2008). *Medicago truncatula* is typically well-colonized by AMF, and can accumulate substantially more biomass when colonized by AMF than when grown in the absence of AMF, particularly under nutrient stress (Watts-Williams et al., 2017). On the other hand, *M. truncatula* can display lowered AMF colonization and neutral growth responses to AMF when nutrients are highly available (Watts-Williams, Jewell, et al., 2019). Almost all physiological studies of *M. truncatula* present growth responses to AMF based on the harvest time point biomass, which may mask temporal responses to AMF.

Typical parameters to assess plant growth over time are low/medium-throughput repeated measures of leaf area (Son & Smith, 1988; Tester, Smith, Smith, & Walker, 1986), and subsequent destructive harvests (Wright, Scholes, & Read, 1998). Clearly, both these methods have disadvantages, as subsequent harvests do not repeatedly measure the

same plant, and leaf area measurements are typically time-intensive. A shoot high-throughput phenotyping (HTP) platform is an advanced tool for studying the aboveground growth of plants through time by daily imaging of the same plant (Berger, Regt, & Tester, 2012). Such HTP platforms have been successfully used in previous experiments studying plant stress responses and genetic variation studies, for example, in barley (Honsdorf, March, Berger, Tester, & Pillen, 2014; Neumann et al., 2015), pea (Humplík et al., 2015), and chickpea (Atieno et al., 2017). It has also been used recently to explore the complex temporal dynamics of mycorrhizal growth responses and interaction with Zn availability, from positive through to negative in three plant species (Watts-Williams, Jewell, et al., 2019), and to explore how mycorrhizal responses are driven by resource allocation (Riley et al., 2019).

Here, we build on previous work using a HTP platform to discover how strong the effect of soil P availability is on *M. truncatula* growth responses to AMF, and whether harvest time point data are consistent with growth responses over the course of the plant's life. Specifically, we were seeking to answer two research questions:

1. Is soil P or Zn availability a stronger driver of plant responses to AMF?
2. Does the nature of shoot growth responses to AMF change over the life of the plant?

2 | MATERIALS AND METHODS

2.1 | Preparation of soil, inoculum, and plant material

The soil used in this study was a 1:9 (*w*/*w*) mixture of field soil and fine sand. The field soil was collected from the Mallala region of South Australia, with bicarbonate-extractable (Colwell) P content of 20.3 mg P kg^{-1}, DTPA-extractable Zn concentration of 0.8 mg Zn kg^{-1}, and pH of 7.4. The soil was sieved to <2 mm to eliminate any coarse debris, autoclaved twice, and oven-dried before being mixed thoroughly with the twice autoclaved sand.

The AMF inoculum used was composed of dry soil, spores, external hyphae of *Rhizophagus irregularis* WFVAM10, and root fragments of colonized Marigold (*Tagetes patula*) plants. The mock inoculum was a mixture of dry soil and root fragments of Marigold plants that had not been inoculated with *R. irregularis*. In the AMF-inoculated pots, 140 g (10% pot weight) of the *R. irregularis* WFVAM10 inoculum was mixed thoroughly with soil to a total of 1,400 g. To the other half of the pots, the mock inoculum was added and mixed in the same procedure.

The soil P treatments were established by adding 2 or 20 mg P kg^{-1} soil in the form of $CaHPO_4$ solution to both mycorrhizal and mock-inoculated pots. These additions resulted in bicarbonate-extractable P contents of 7.95 and 28.1 mg P kg^{-1}, respectively, and are referred to as P2 and P20 hereafter. The soil Zn treatments were established by adding 0, 2, 5, 15, and 25 mg Zn kg^{-1} in the form of $ZnSO_4.7H_2O$ solution to the pots. The soils had diethylenetriaminepentaacetic acid (DTPA)-extractable Zn concentrations of: 0.3, 1.1, 4.6, 8.8, and 19.9 mg Zn kg^{-1}, respectively. These five Zn treatments are referred to as Zn0, Zn2, Zn5, Zn15, and Zn25 hereafter. The thoroughly mixed soil was used to fill free-draining plastic pots (180 mm height, 90 mm width and length, 0.97 L volume). Each treatment was replicated six times, giving a total of 120 pots.

Medicago truncatula L. cv. Jemalong A17 seeds were scarified using sand paper, then sterilized in 10% sodium hypochlorite (bleach) solution for 3 min. After thoroughly rinsing with reverse osmosis (RO) water, the seeds were placed on moist filter paper in Petri dishes and then sealed and placed at 4°C for 72 hr. The Petri dishes were then incubated at 25°C for 24 hr then unsealed and left at room temperature, in the natural light for 4 days before being transplanted to the previously prepared pots, with one plant per pot. From the second week, all plants received 10 ml of modified Long Ashton solution (omitting P and Zn) on a weekly basis. In addition, the Medicago plants each received a total of 50 mg N as NH_4NO_3 over the course of the experiment (10 mg N $plant^{-1}$ at 2, 7, 13, 27, and 41 days after planting [DAP], respectively) in order to suppress nodulation.

2.2 | Plant growth, harvest, and sample analysis

Planting occurred on 25 July 2018 (denoted 0 DAP) in two phases. Firstly, the plants were grown in a glasshouse on The Waite Campus of The University of Adelaide, Australia. The temperature in the glasshouse ranged between 14.2 and 24.4°C (night to day) with supplemental lighting in a 16/8 day/night photoperiod. Plants were watered daily with 30 ml of RO water. Then, from 14 DAP, plants were loaded onto the phenotyping cart system in the Smarthouse of The Plant Accelerator, Australian Plant Phenomics Facility, located at the University of Adelaide's Waite campus until harvest. Temperatures in the Smarthouse during the growing period ranged from 14.7 to 29.2°C, with natural day length and light levels. The plants were watered gravimetrically with RO water (to 10% (g/g) soil weight) by the automatic system on a daily basis. Imaging was taken daily from 22 to 53 DAP.

All plants were destructively harvested at 53 DAP, at the onset of flowering, as follows. Plant shoots were cut at soil level and the fresh weights of shoots were taken. Plant roots were washed free of any attached soil with RO water and fresh weights of total and remaining roots were measured before a subsample of fresh root biomass was cut and placed into 50% ethanol. The shoot and remaining root samples were then dried in an oven at 60°C for 72 hr before dry weights were determined.

In order to quantify the elemental concentration of the shoots, the dried shoots were ground finely to homogenize the sample, and approximately 200 mg subsamples were digested in 2 ml nitric acid and 0.5 ml hydrogen peroxide. Diluted plant digests were then analyzed for concentrations of P and Zn by inductively coupled plasma optical emission spectroscopy (ICP-OES) (Thermo Jarrell Ash Corp.).

Fresh roots fixed in ethanol for 48 hr were rinsed with RO water and then cleared in 10% potassium hydroxide at room temperature for 7 days. Cleared roots were rinsed and then stained in 5% ink in vinegar (modified from Vierheilig, Coughlan, Wyss, and Piche (1998))

at 60°C for 10 min before being destained in acidified water for 24 hr, then washed and moved to 50% glycerol solution for storage. Mycorrhizal colonization was determined on stained root samples according to the gridline intersect method (Giovannetti & Mosse, 1980).

2.3 | Shoot phenotyping data collection and preparation

The experiment comprised all combinations of two AMF treatments (mock inoculation and AMF inoculation), two levels of soil P addition (2 and 20 mg P kg^{-1} soil), and five levels of soil Zn addition (0, 2, 5, 15, and 25 mg Zn kg^{-1} soil), resulting in a total of 2 × 2 × 5 = 20 treatments. There were six biological replicates, resulting in a total of n = 120 plants. The pots were organized in the northeast Smarthouse in a split-plot design (see Figure S1), with each block of a replicate (i.e., one pot of each treatment; 20 pots total) occupying 10 positions in two adjacent lanes. Within each block, the 10 nutrient treatments were randomized using part of a Latin-square design, to pairs of pots in adjacent lanes, and the two AMF treatments were then randomized within that pair. The design was randomized using dae (Brien, 2019b) packages for the R statistical computing environment (R Core Team, 2018).

From the images, the projected shoot area (PSA) of the plant, as viewed using an RGB camera, was obtained; more precisely, PSA was calculated as the sum of the areas as measured (in kilopixels) from four camera views, comprising three (non-orthogonal) side views and a view from above. At the conclusion of the experiment, it was found that two of the plants had died; a third plant was removed as an outlier. Thus, the analysis dataset comprised n = 120−3 = 117 plants.

2.4 | Data preparation

The imaging data were prepared using the package growthPheno (Brien, 2019c) for the R statistical computing environment (R Core Team, 2019). The PSA absolute growth rate (AGR) describes the estimated shoot biomass accumulated over a defined number of days, and PSA relative growth rate (RGR) describes the growth relative to the previous day, over a defined number of days. Both AGR and RGR are calculated from the PSA values by differencing consecutive PSA and ln(PSA) values, respectively, and dividing by the time differences. The PSA growth curve was loess smoothed at a value of five degrees of freedom (moderate smoothing).

After examination of the plots for the smoothed traits sPSA, sPSA AGR, and sPSA RGR, it was decided to investigate growth with respect to the time points 20, 30, 35, 40, and 53 DAP. A total of 11 traits were analyzed as follows:

- Single-day responses: sPSA for 20, 30, 35, 40, and 53 DAP;
- Interval responses: sPSA AGR and sPSA RGR for each of the DAP intervals 20–30, 30–40, and 40–53;

Each of these traits yielded a single value for each plant.

2.5 | Statistical analysis

To produce phenotypic predictions, a mixed model analysis was performed for each trait using the R package ASReml-R (Butler, Cullis, Gilmour, Gogel, & Thompson, 2018) and asremlPlus (Brien, 2019a), packages for the R statistical computing environment (R Core Team, 2019). The maximal mixed model for this analysis was of the form

$$y = X\beta + Zu + e,$$

where y is the response vector of values for the trait being analyzed; β is the vector of fixed effects; u is the vector of random effects; and e is the vector of residual effects. X and Z are the design matrices corresponding to β and u, respectively. The fixed-effect vector β was partitioned as $\beta^{\top} = \left[\begin{array}{cccccccc} \mu\beta_R^{\top} & \beta_M^{\top} & \beta_P^{\top} & \beta_Z^{\top} & \beta_{M:P}^{\top} & \beta_{M:Z}^{\top} & \beta_{P:Z}^{\top} & \beta_{M:P:Z}^{\top} \end{array}\right]$, where μ is the overall mean and the β subvectors correspond to the respective effects of replicates (R); main effects Mycorrhiza (*Mycorrhiza*), Phosphorus (*P*), and Zinc (*Zn*) of the treatment factors; two-way treatment interactions (*Mycorrhiza:P*, *Mycorrhiza:Zn*, and *P:Zn*); and three-way interaction (*Mycorrhiza:P:Zn*). Thus, the first β subvector captured spatial variation within the Smarthouse, while the remaining subvectors captured treatment effects. The random effects vector u captured any non-trend spatial variation associated with differences between main-plots within replicates.

The residual effects e were assumed to be normally distributed, with a variance σ^2, which may have varied with both *P* and *Mycorrhiza*. We assumed for illustrative purposes that the dataset comprised n = 120 observations ordered first by *P* (with levels L and H) and then by *Mycorrhiza* (levels − and +), yielding 2 × 2 = 4 consecutive subgroups each comprising 5 × 6 = 30 plants. Then, the residuals were modeled as

$$N\left(0_{120}, \left[\begin{array}{cccc} \sigma_L^2 - I_{30} & 0_{30} & 0_{30} & 0_{30} \\ 0_{30} & \sigma_L^2 + I_{30} & 0_{30} & 0_{30} \\ 0_{30} & 0_{30} & \sigma_H^2 - I_{30} & 0_{30} \\ 0_{30} & 0_{30} & 0_{30} & \sigma_H^2 + I_{30} \end{array}\right]\right),$$

where I_{30} and 0_{30} denoted identity and zero matrices, respectively. For each trait, a likelihood ratio test was used to determine whether the variance model could be simplified by the removal of mycorrhizal difference (i.e., a difference in P only, modeled using two variance parameters σ_L^2 and σ_H^2). While the full four-parameter variance model was found to be warranted for most sPSA, sPSA AGR, and harvest traits, it could be simplified to a difference for P only for three traits. A simplified version of this model was used to analyze the mycorrhizal colonization data for the AMF-inoculated plants. Residual versus fitted values and normal probability plots were produced for all analyses to check that the analysis assumptions were met and those for the final analyses were found to be satisfactory in all cases. Wald F-statistics were produced for the treatment main effects and interactions, and these were used to identify models that described the effects of the treatment factors on the traits. The selected models were used to obtain phenotypic

predictions (BLUEs), and their standard errors, from which least significant pairwise difference (LSD) values were obtained.

3 | RESULTS

3.1 | Growth analysis revealed complex treatment interactions that changed over time

Raw PSA values displayed considerable day-to-day variation, which has been observed in other imaging experiments. These variations can lead to a significant amount of noise when calculating AGR and RGR on a daily basis from the raw values. Spline smoothing was therefore used to smooth the PSA values and to calculate the AGR and RGR from smoothed PSA (sPSA) predictions. As previously noted, the smoothed values of PSA with five degrees of freedom were utilized to evaluate the growth over time. Descriptive plots based on daily sPSA values were constructed for each *P* treatment, smoothed across treatment replicates, and split by *Mycorrhiza* treatment or by *Zn* treatment (Figure S2).

For the sPSA traits, the significant interaction between *P* and *Zn* persisted throughout the chosen DAPs for analysis (Table S1). This was mainly driven by the Zn0 treatment, which behaved differently depending on soil P availability: at low P, the Zn0 treatment consistently returned the highest sPSA, while at high P it had the lowest sPSA (Figure 1). In addition, there was a significant effect of *Mycorrhiza* at each DAP, either through interaction with *P* (35, 40, and 53 DAP) or as a main effect (20 and 30 DAP). The sPSA predictions indicate that the mycorrhizal plants were larger than the non-mycorrhizal plants irrespective of P supply at 20 and 30 DAP; this switched to an interaction with *P* for 40 DAP, whereby the mycorrhizal plants had a greater sPSA only in the low P treatment.

The model predictions for the sPSA AGR describe the estimated shoot biomass accumulated over a defined time period (Figure 2). For the three sPSA AGR time periods defined in this experiment, each had an interaction between *Mycorrhiza* and *P*, and also between *P* and *Zn*. The *Mycorrhiza***P* interaction was similar to that observed for the sPSA traits: at the P2 level, the mycorrhizal plants consistently grew faster that the non-mycorrhizal plants, whereas at the P20 level, the AMF effect shifted from positive at the earliest time period (20–30 DAP) to negative in the final time period (40–53 DAP). Likewise, the *P***Zn* interactions observed in the AGR intervals were similar to that in sPSA traits, whereby the Zn0 treatment had the greatest AGR at P2, but the lowest at P20.

The model predictions for the sPSA RGR describe the growth relative to the previous day, over a defined time period. For sPSA RGR, the interaction between *Mycorrhiza* and *P* affected all three time periods examined. This was due to AMF colonization affecting RGR differently to the non-colonized plants depending on soil P availability (Figure 3). For example, at the first time period, the mycorrhizal plants have a greater RGR than the non-mycorrhizal plants at P2, but RGR is matched at P20. Conversely, during the last time period, the RGR of the mycorrhizal plants are matched with the non-mycorrhizal plants at P2, but the non-mycorrhizal plants have a greater RGR at P20. These complex interactions between RGR with time period are further reflected in other significant terms: at 20–30 DAP, there was an interaction between *P* and *Zn*, which followed the same pattern as sPSA and AGR, with the Zn0 treatment had the greatest RGR at P2, but the lowest at P20. At 30–40 DAP, there was an interaction between *Mycorrhiza* and *Zn*, whereby the AMF-colonized plants only had a greater RGR at Zn25 than at Zn0 or Zn5.

3.2 | Soil P availability affected mycorrhizal colonization

In the AMF-inoculated plants, at the time of harvest, the roots were well-colonized across all the P and Zn addition treatments (Table 1). There was no significant interaction between *Mycorrhiza* and *Zn*; only *P* was significant (Table S2), mycorrhizal colonization was higher in the P2 plants than the P20 plants, irrespective of Zn treatments (predictions: 60.1 ± 2.6% and 37.4 ± 2.0%, respectively; LSD (5%): 6.7%). In the mock-inoculated plants, no mycorrhizal colonization was observed in the roots (data not shown).

3.3 | Nutrient availability and AMF interacted to affect final plant biomass and nutrition

For shoot dry weight, there were significant two-way interactions between *Mycorrhiza* and *P* and also between *P* and *Zn* (Table S2). The predictions under this model show that shoot dry weight at P20 was higher than P2 in all plants, except those for the mycorrhizal P2 plants at Zn0 (Figure 4). Furthermore, at P2, but not at P20, shoot dry weight was higher in the mycorrhizal plants. The root dry weight data exhibited the same statistical differences as the shoot dry weight data.

For shoot P concentration, all three two-way interactions between *Mycorrhiza*, *P*, and *Zn* were significant (Table S2). The shoot P concentration in the P20 plants was higher than in the P2 plants, across all *Zn* levels (Figure 5a). Within the P20 plants, (a) the shoot P concentration of the Zn0 plants was greater than that of the Zn2, Zn15, and Zn25 plants and (b) the mycorrhizal plants had higher shoot P concentration than the non-mycorrhizal plants, for all Zn levels except Zn25. Within P2 plants, there were no significant differences between mycorrhizal and non-mycorrhizal plants and little difference between Zn levels.

For shoot Zn concentration, there was an interaction between *Mycorrhiza* and *P* and a main effect of *Zn* (Table S2). Figure 5b shows that, for all plants, as added Zn increased so did shoot Zn concentrations. It can also be seen that the only differences in shoot Zn concentrations between mycorrhizal and non-mycorrhizal occurred with P20 plants.

4 | DISCUSSION

4.1 | Phosphorus is a strong driver of mycorrhizal growth responses

The arbuscular mycorrhiza literature has consistently and historically reported on the strong effects of soil P availability on traits such as

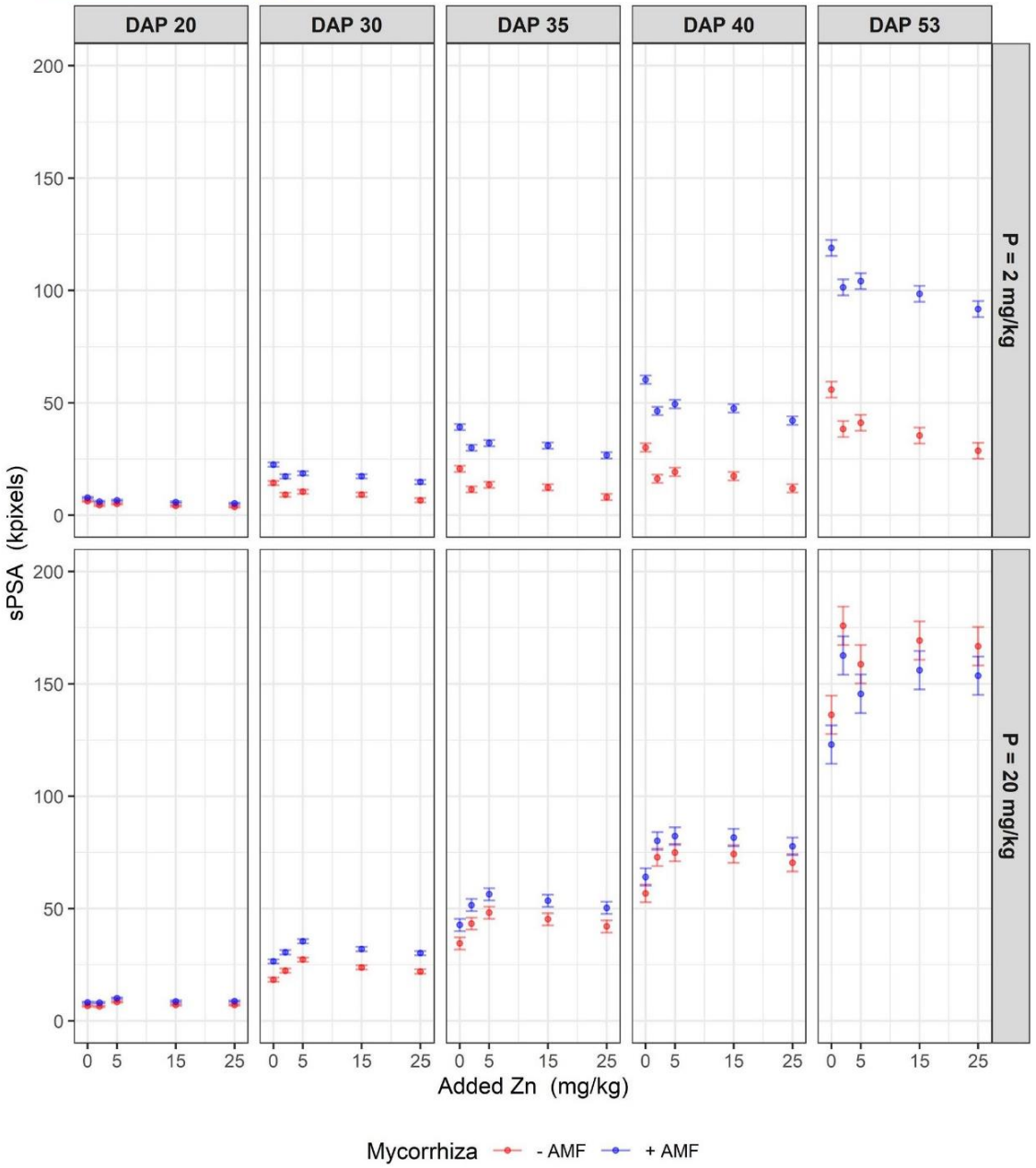

FIGURE 1 Predicted PSA at defined DAP of *Medicago truncatula* inoculated with the AMF *Rhizophagus irregularis* (blue) or mock-inoculated (red), grown at two soil P concentrations and five soil Zn concentrations. The error bars correspond to predictions ±1/2 the least significant pairwise difference at the conventional α = 0.05 significance level (5% half-LSD). See Table S1 for ANOVA results. AMF, arbuscular mycorrhizal fungi; DAP, days after planting; PSA, projected shoot area

mycorrhizal colonization of roots and the activity of the mycorrhizal P uptake pathway (Chiu & Paszkowski, 2019). The present study is no exception to this, and the stark differences in plant responses to AMF when P was supplied at 2 mg compared with 20 mg P kg^{-1} soil are evident throughout the growth data, both over time and at the final harvest.

Clearly, there was a strong biomass response to AMF when the *M. truncatula* plants were grown at low P, both in the shoot and

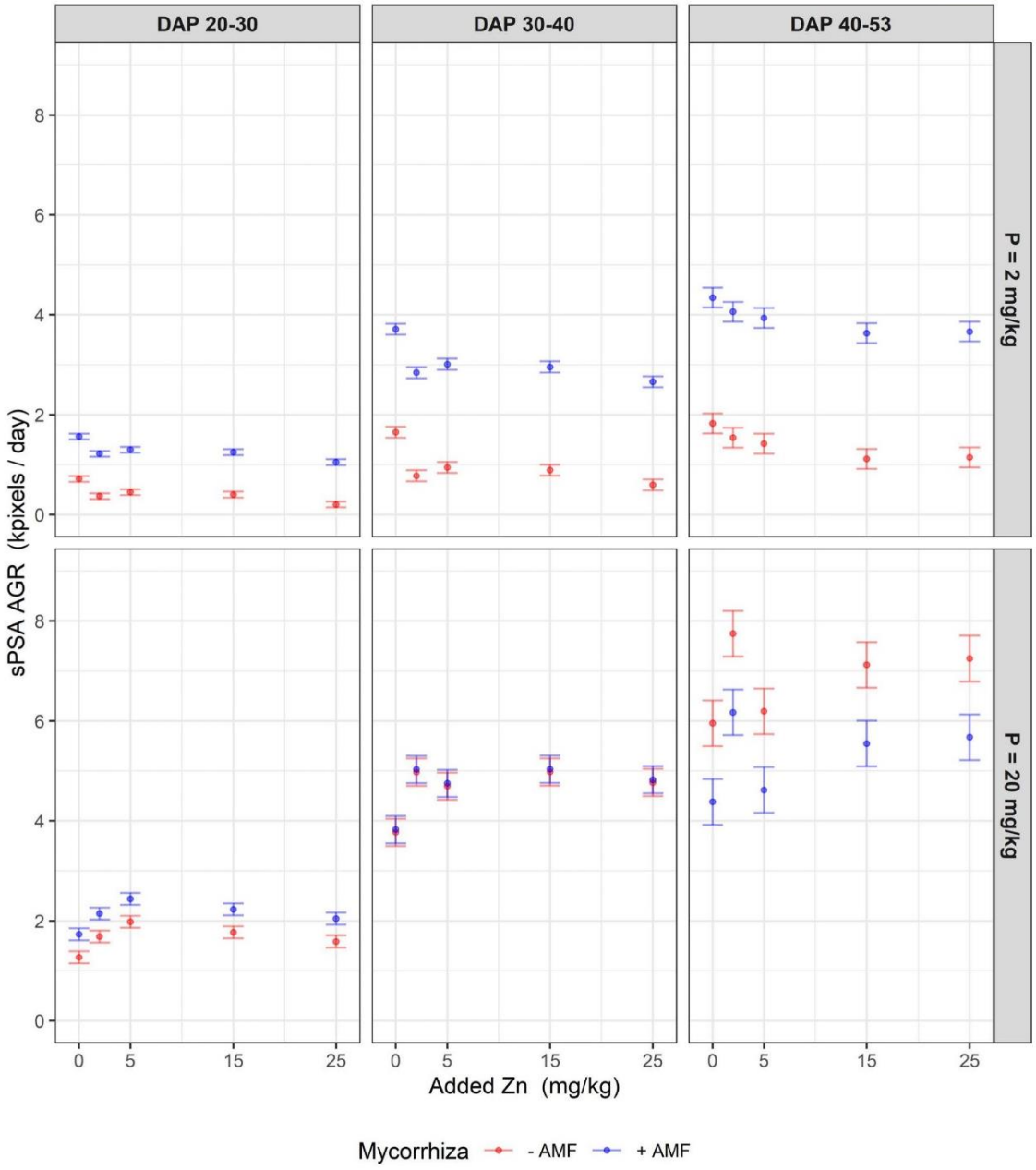

FIGURE 2 Predicted PSA AGR at defined time intervals of *Medicago truncatula* inoculated with the AMF *Rhizophagus irregularis* (blue) or mock-inoculated (red), grown at two soil P concentrations and five soil Zn concentrations. The error bars correspond to predictions ±1/2 the least significant pairwise difference at the conventional $\alpha = 0.05$ significance level (5% half-LSD). See Table S1 for ANOVA results. AMF, arbuscular mycorrhizal fungi; AGR, absolute growth rate; PSA, projected shoot area

root biomass at harvest. When the soil was amended with a high amount of P, however, the AMF growth response became neutral. This trend was also observed in the phenotyping data, with AGR and RGR values higher in the mycorrhizal plants only at low soil P availability. In terms of harvest point biomass data, this neutralization of response to AMF with high P has been demonstrated previously in this plant species (Jakobsen et al., 2016; Watts-Williams, Jewell, et al., 2019). Some plant species respond neutrally to

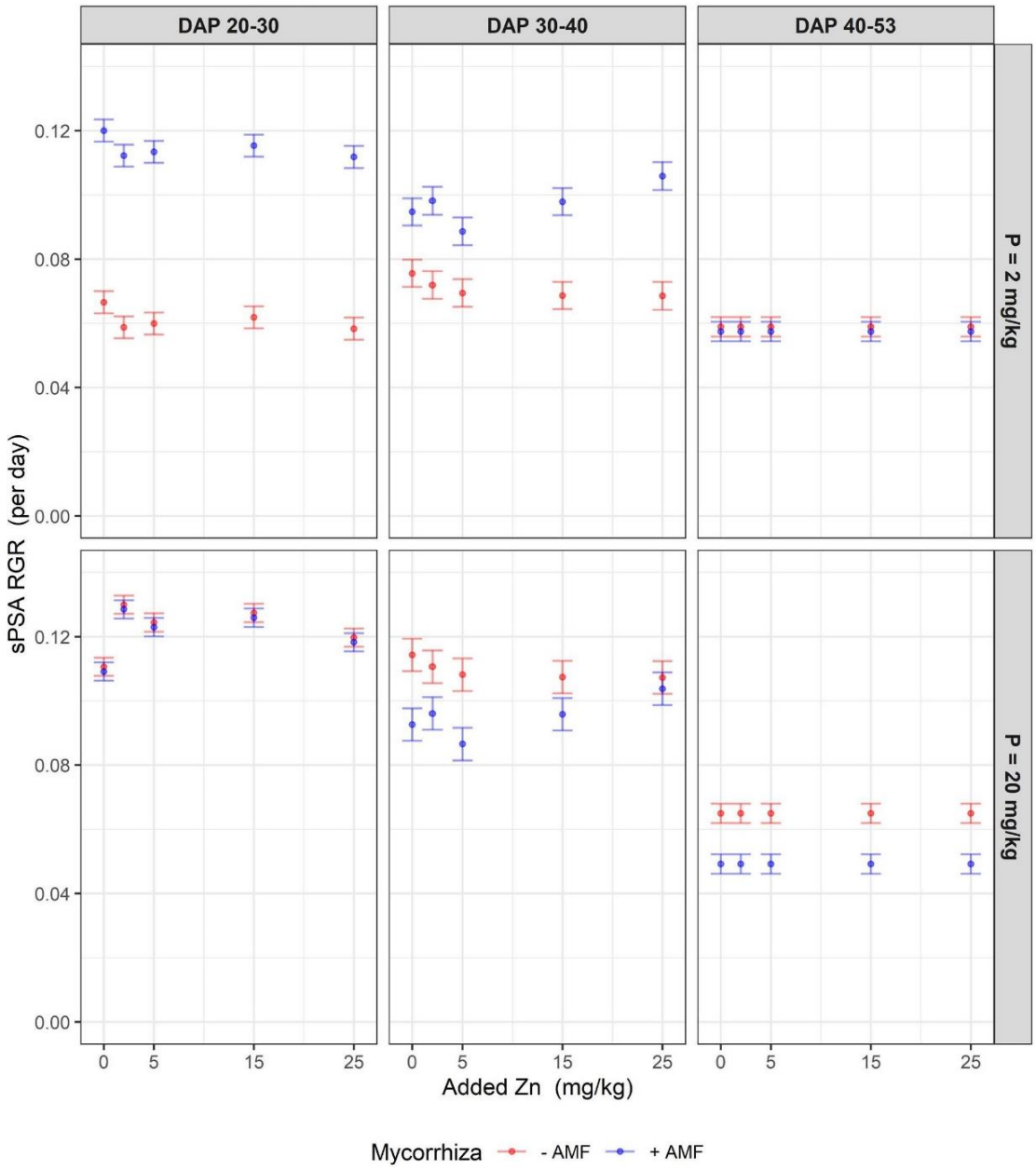

FIGURE 3 Predicted PSA RGR at defined time intervals of *Medicago truncatula* inoculated with the AMF *Rhizophagus irregularis* (blue) or mock-inoculated (red), grown at two soil P concentrations and five soil Zn concentrations. The error bars correspond to predictions ±1/2 the least significant pairwise difference at the conventional $\alpha = 0.05$ significance level (5% half-LSD). See Table S1 for ANOVA results. AMF, arbuscular mycorrhizal fungi; PSA, projected shoot area; RGR, relative growth rate

colonization by AMF regardless of the soil P concentration, such as wheat, barley, and tomato (Grace, Smith, & Smith, 2009; Watts-Williams et al., 2013). On the other hand, there are instances where AMF colonization still confers a biomass advantage even when soil P availability is high, such as in maize (Jansa, Mozafar, & Frossard, 2003; Kothari, Marschner, & Römheld, 1991). Clearly, the nature of AMF growth response (positive, neutral, negative) with high P supply is strongly influenced by the host plant identity

TABLE 1 Mycorrhizal colonization (percent root length colonized) at harvest of *Medicago truncatula* inoculated with the AMF *Rhizophagus irregularis* and grown at two soil P availabilities and five different soil Zn availabilities ranging from no addition of Zn (Zn0) to high soil Zn addition (Zn25)

	P2		P20		
	prediction	SE	prediction	SE	LSD (5%)
Zn0	65.86	5.83	33.61	4.47	14.83
Zn2	55.26	5.83	35.98	4.47	14.83
Zn5	61.85	6.43	43.97	4.47	15.81
Zn15	53.68	5.83	41.85	4.47	14.83
Zn25	63.65	5.83	31.47	4.47	14.83

Abbreviation: AMF, arbuscular mycorrhizal fungi.

(genotype) (Watts-Williams, Cavagnaro, & Tyerman, 2019). The low P soil concentration used in this experiment was limiting to plant growth, but the *M. truncatula* plants did not display other visual symptoms of P deficiency (e.g., foliar purple coloring); however, this species and ecotype of pasture legume are known to be very responsive to *R. irregularis* inoculation (Watts-Williams, Cavagnaro, et al., 2019; Watts-Williams et al., 2017), so the poor growth without AMF inoculation even when there was some P added to the soil is not surprising. It should be noted that there are, inevitably, limitations in interpreting the results due to the substrate used in the experiment. The greater percent root length colonized by AMF at low P compared with high P is also consistent with the literature (Bolan, Robson, & Barrow, 1984; Liu, Hamel, Hamilton, Ma, & Smith, 2000), and points to a more involved role for AMF in P uptake at low P compared to high P.

It is important to note that although the biomass response to AMF was clearly dependent on the availability of soil P, the concentration of P in the shoots was higher in the mycorrhizal plants at both low and high P. This has been seen previously (Smith et al., 2004), and suggests that the activity of the mycorrhizal P uptake pathway is greater than the observed improvement in plant biomass alone (Smith, Smith, & Jakobsen, 2003).

4.2 | Plant growth responses to mycorrhizal fungi are temporal in nature

The biomass and nutrient responses at the time of harvest of *M. truncatula* in this study were consistent with previous work. However, the novelty of this study lies in the HTP and thus the temporal analysis of *M. truncatula*'s response to AMF and soil P and Zn availabilities. The HTP analysis revealed patterns of growth in response to AMF that would have been masked by analyzing the final harvest data alone.

Even at 35 DAP, the mycorrhizal plants were larger than the non-mycorrhizal plants in both P treatments, and it was only from 40 DAP that the shoot growth patterns diverged depending on soil P supply. From that point, the positive AMF growth response increased in magnitude with time in the plants supplied with low P, while the plants growing at high P were matched until harvest. The AMF contribution to growth over the life of the plants grown at high P could easily be underestimated based on harvest point data—even though the mycorrhizal plants were larger 18 days prior to harvest. The patterns in sPSA at high P, notably the divergence after 35 DAP, can be further explained by the AGR data, indicating a switch from higher AGR in the mycorrhizal plants at the start of the experiment to higher AGR in the non-mycorrhizal plants at the end of the experiment.

To understand the mechanisms underlying the observed changes in biomass accumulation over the life of a *M. truncatula* plant, it is necessary to consider how the development of the mycorrhizal association changes over its life, and then how the contribution by the mycorrhizal P uptake pathway may also be modulated. Root growth can be slow initially due to the C cost as the plant establishes leaf area (photosynthetic capacity), but as C supply is secured, root growth and soil exploration can increase (Eissenstat & Volder, 2005). Establishment of mycorrhizal colonization of roots starts from about 10 DAP (Bruce, Smith, & Tester, 1994; Carling, Brown, & Brown, 1979; Manjunath & Bagyaraj, 1981), depending on the species of plant and colonizing AMF, and soil P availability, but once established, external hyphal growth can explore a greater volume of soil than roots (Sanders & Sheikh, 1983). There is likely a lag phase between establishment of the mycorrhizal symbiosis and a measurable change in biomass (Watts-Williams, Emmett, et al., 2019); in the present experiment, this appears to be until at least 20 DAP.

The interplay between the two root phosphate uptake pathways—the mycorrhizal (MPU) and the direct (DPU)—is a likely driver behind the temporal growth responses to AMF (Grønlund et al., 2013; Smith, Jakobsen, Grønlund, & Smith, 2011). The activity of the MPU is clearly driving the increasing AMF growth response over time in the low P soil. The DPU may be also contributing at low soil P, with the simultaneous activity of the MPU and DPU working to confer an advantage over the DPU alone, as demonstrated previously in radioisotope tracing studies (Smith et al., 2004; Watts-Williams, Jakobsen, Cavagnaro, & Grønlund, 2015). In the high P soil, however, the relationship between the two uptake pathways over time must be more complex; we postulate that once colonization had established, the MPU predominated in the first half of the experiment to confer the observed AMF growth response, but once the root system had established, the DPU was activated, and either suppressed the MPU, explaining the lower cumulative mycorrhizal colonization, or worked in concert with the MPU to maintain (but not increase) AGR. In the non-colonized plants, where only the DPU was active, there is a clear advantage at high P availability in terms of growth rate over the mycorrhizal plants in the final stage of the experiment. This high growth rate in the non-mycorrhizal plants toward the end of the experiment may be explained by differences in the timing of peak growth rate (and perhaps maturity) for mycorrhizal versus non-mycorrhizal plants; a higher and slightly later peak AGR was also observed in the non-mycorrhizal plants when supplied with high soil P, in a previous HTP experiment (Riley et al., 2019).

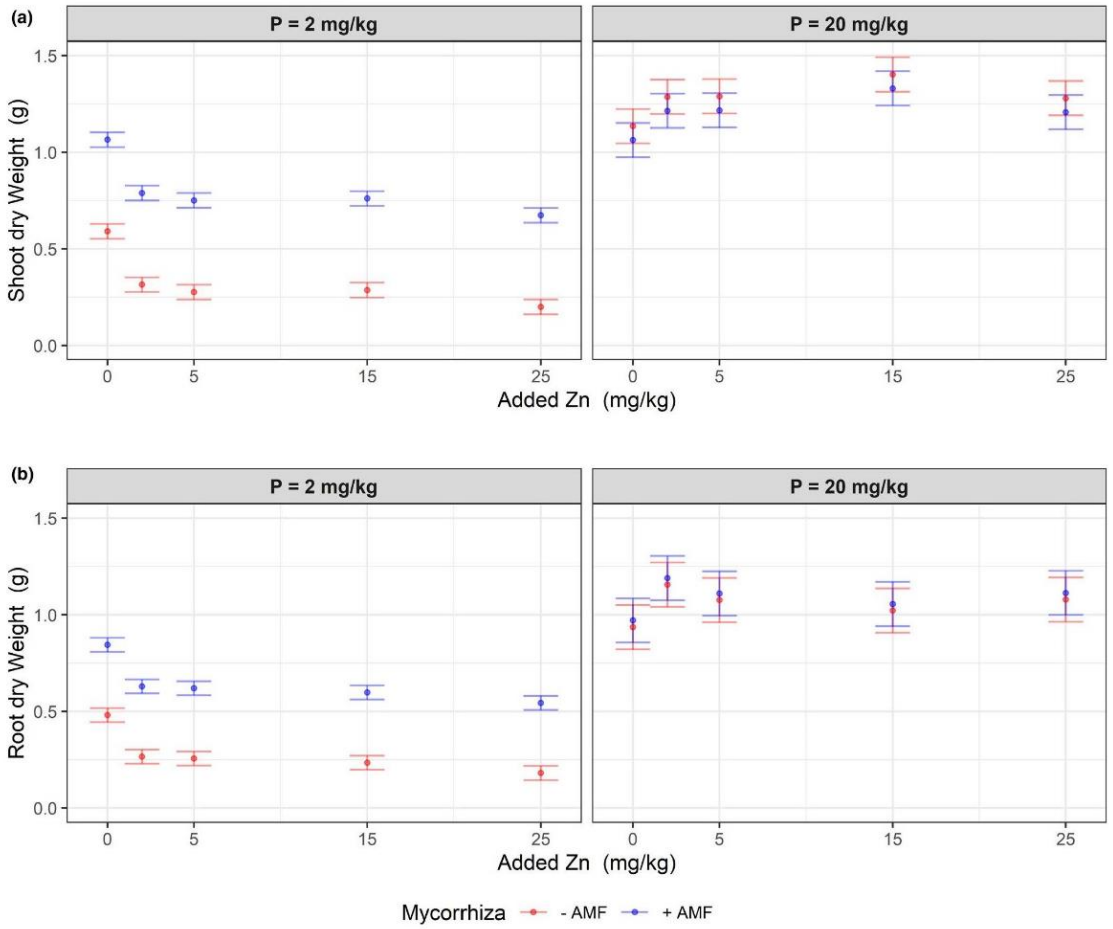

FIGURE 4 Shoot dry weights (a) and root dry weights (b) at harvest of *Medicago truncatula* inoculated with the AMF *Rhizophagus irregularis* or mock-inoculated, grown at two soil P concentrations and five soil Zn concentrations. The error bars correspond to predictions ±1/2 the least significant pairwise difference at the conventional $\alpha = 0.05$ significance level (5% half-LSD). See Table S2 for ANOVA results. AMF, arbuscular mycorrhizal fungi

The difference in late-stage AGR between mycorrhizal treatments at P20 also explains the harvest data that show the non-mycorrhizal plants were slightly larger than the mycorrhizal plants on a total weight basis.

Although the results would be incredibly useful, it would be highly laborious to measure both growth and P uptake pathway activity (with radioisotopes) over time, simultaneously. There is literature on radioisotope labeling studies that measure the activity of the two pathways at different times through destructive harvests (Grønlund et al., 2013; Smith et al., 2004), and only a handful of studies that have measured plant growth responses to AMF over time with low- or high-throughput methods (Son & Smith, 1988; Tester et al., 1986). One proxy may be to use high-throughput shoot phenotyping and take repeated measures of the mycorrhiza-induced P transporter gene, *MtPT4*, in the roots. Previous studies have shown that *MtPT4* (or relevant orthologue in other species) expression correlated with MPU activity (Grace, Cotsaftis, et al., 2009; Watts-Williams et al., 2015), while others did not (Sawers et al., 2017). So, if the samples could be taken without being too invasive or destructive (e.g., via soil coring), *MtPT4* expression may give an indication of the activity of the MPU over time, but the correlation between *MtPT4* and MPU activity would first need to be confirmed in an additional experiment testing that hypothesis.

4.3 | Zinc was not in itself a strong driver of mycorrhizal growth responses

It is well established that there are strong interactions between P and Zn in the soil, during plant and fungal uptake (Jansa et al., 2003; Lambert, Baker, & Cole, 1979; Zhu, Smith, & Smith, 2001), and in the plant (Loneragan, Grove, Robson, & Snowball, 1979; Loneragan &

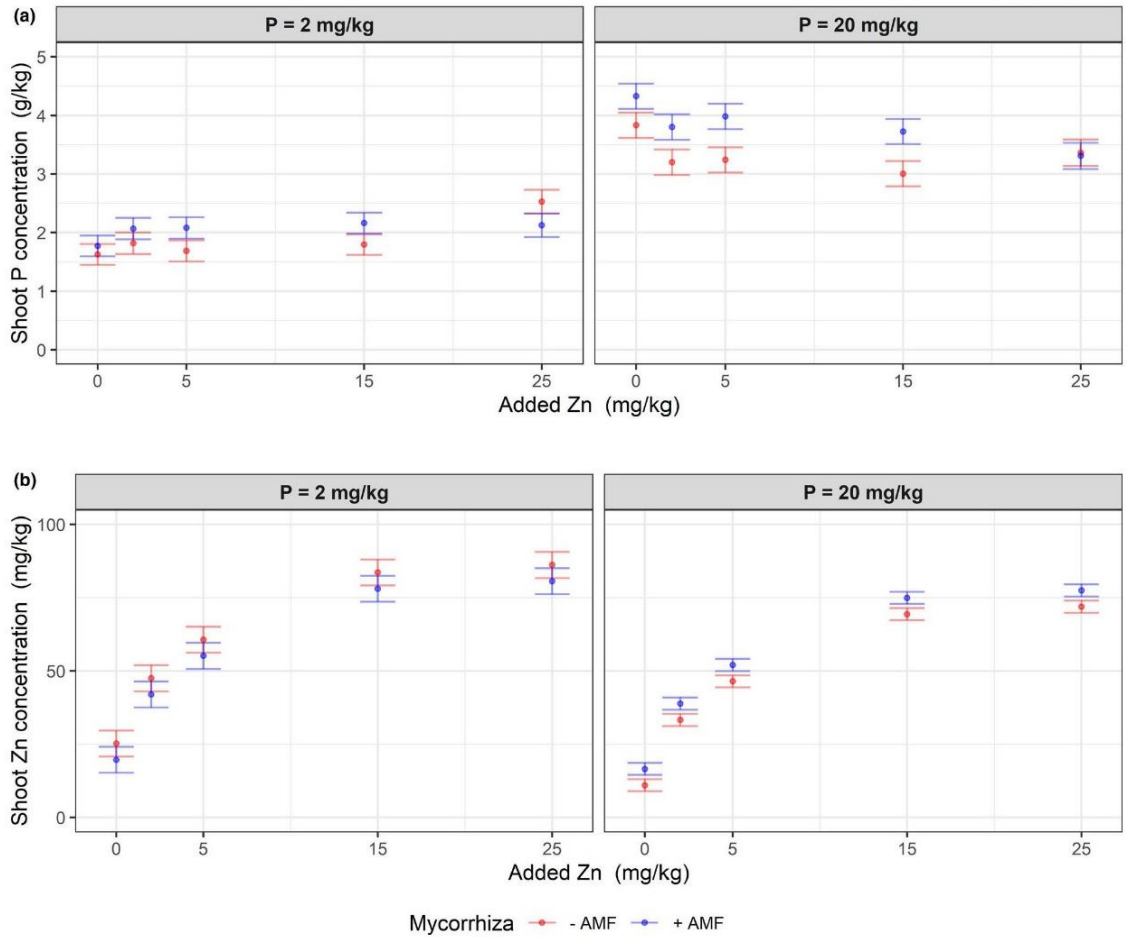

FIGURE 5 Shoot P (a) and shoot Zn (b) concentrations at harvest of *Medicago truncatula* inoculated with the AMF *Rhizophagus irregularis* or mock-inoculated, grown at two soil P concentrations and five soil Zn concentrations. The error bars correspond to predictions ±1⁄2 the least significant pairwise difference at the conventional $\alpha = 0.05$ significance level (5% half-LSD). See Table S2 for ANOVA results. AMF, arbuscular mycorrhizal fungi

Webb, 1993; Robson & Pitman, 1983). The treatments in this study were established to examine how the three-way interaction between Zn, P, and AMF manifested in plant growth over time. However, no three-way interactions were observed for any of the growth or nutrition variables, which is in contrast to other studies that found the combined availability of P and Zn in the soil affects plant growth responses to AMF (Watts-Williams & Cavagnaro, 2012; Nguyen et al., 2019). Instead, the interaction between P and Zn predominated, and in most cases, AMF effects on plant growth were hardly influenced by soil Zn concentration. This may be because *M. truncatula* is more sensitive to low P availability (Burleigh & Harrison, 1999; Tang, Hinsinger, Drevon, & Jaillard, 2001) than to the availability of Zn in the soil.

The interaction between P and Zn had the most noticeable effects when no Zn was added to the soil (Zn0); when P availability was low, the lowest soil Zn availability conferred the greatest shoot biomass and growth rate of all the Zn treatments. This trend appeared by 30 DAP, remained until harvest, and was not influenced by AMF inoculation. In this case, the *M. truncatula* plants may have been limited by P uptake so much that any extra Zn in the soil caused stress through toxicity in plant tissues. We can see that as Zn concentration in the soil increased, shoot Zn concentration also increased substantially, being four to five times higher in the Zn25 treatment than Zn0. Meanwhile, shoot P concentration remained stable across Zn treatments, which suggests that plant growth was negatively affected by the increasing ratio of Zn:P in the shoots. Meanwhile, in the fertilized P treatment, the plants grown in the unamended Zn treatment had the lowest biomass and growth rate of all the Zn treatments. Here, the plants were likely limited by Zn given that the high soil P concentration led to greater biomass accumulation and thus likely a greater demand for micronutrients. In the non-mycorrhizal plants, the shoot Zn concentrations were lower in the

high P treatment than at low P, which supports the conjecture that Zn was limiting growth there.

5 | CONCLUSIONS

To answer our proposed research questions, the results have demonstrated quite clearly that soil P availability is a stronger driver of mycorrhizal growth responses in *M. truncatula*, than is soil Zn availability. We have also demonstrated here that the nature of shoot growth responses to AMF inoculation (i.e., positive, neutral, or negative) do change over the life of the plant; in fact, the harvest time point data for plant biomass in response to AMF inoculation can be very different from what occurs over the life of the plants.

For many research questions, harvest data are sufficient to address the underlying hypotheses; however, with regard to arbuscular mycorrhizal associations, it is important to elicit temporal responses to uncover the underlying mechanisms behind growth responses to AMF, and plant uptake of P. The societal benefit of this work comes from the improvement of our understanding of the function of arbuscular mycorrhizal associations that could lead to better management (i.e., through fertilizer or AMF inoculant application) of crops with the aim to promote positive plant growth responses to AMF colonization.

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AUTHOR CONTRIBUTION

SJWW designed the experiment, interpreted the data, and prepared the first full draft of the manuscript. BTTT participated in experimental design, sample and data analyses, and preparation of the manuscript. NJ and CB participated in experimental design, analyzed the data, and prepared the relevant parts of the manuscript. TRC and BB participated in the experimental design, data interpretation, and critical review of the manuscript.

ORCID

Chris Brien https://orcid.org/0000-0003-0581-1817
Stephanie J. Watts-Williams https://orcid.org/0000-0003-3467-0662

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Plants, People, Planet **Supporting Information**

Article title: High-throughput phenotyping reveals growth of Medicago truncatula is positively affected by arbuscular mycorrhizal fungi even at high soil phosphorus availability.

Authors: B.T.T. Tran, T.R. Cavagnaro, N. Jewell, C. Brien, B. Berger, S.J. Watts-Williams

The following Supporting Information is available for this article:

Table S1 Statistical outcomes for smoothed PSA traits.

Table S2 Statistical outcomes for harvest time point variables.

Fig. S1 Experimental design for pot placement within Smarthouse.

Fig. S2 Smoothed PSA over the course of the experiment.

Table S1. For smoothed PSA traits, summaries for the *P*-values for the Wald F-statistics that test the chosen model for the four interactions of the three treatment factors (*Myc:P:Zn, Myc:P, Myc:Zn and P:Zn*) and their main effects (*Mycorrhiza*, *P* and *Zn*). Statistically significant terms ($p \leq 0.05$) are in bold. *Myc*: mycorrhiza, *P*: phosphorus, *Zn*: zinc.

Trait + DAP	*Myc*P*Zn*	*Myc*P*	*Myc*Zn*	*P*Zn*	*Myc*	*P*	*Zn*
sPSA 20	0.587	0.109	0.106	**0.008**	**<0.001**	na	na
sPSA 30	0.460	0.150	0.149	**<0.001**	**<0.001**	na	na
sPSA 35	0.377	**0.001**	0.150	**<0.001**	na	na	na
sPSA 40	0.358	**<0.001**	0.144	**<0.001**	na	na	na
sPSA 53	0.228	**<0.001**	0.482	**<0.001**	na	na	na
sPSA AGR 20-30	0.322	**0.005**	0.162	**<0.001**	na	na	na
sPSA AGR 30-40	0.454	**<0.001**	0.158	**<0.001**	na	na	na
sPSA AGR 40-53	0.126	**<0.001**	0.843	**0.032**	na	na	na
sPSA RGR 20-30	0.268	**<0.001**	0.345	**<0.001**	na	na	na
sPSA RGR 30-40	0.319	**<0.001**	**<0.001**	0.195	na	na	na
sPSA RGR 40-53	0.127	**0.001**	0.295	0.312	na	na	0.106

Table S2. For harvest time point response variables, summaries for the *P*-values for the Wald F-statistics that test the chosen model for the four interactions of the three treatment factors (*Myc:P:Zn, Myc:P, Myc:Zn and P:Zn*) and their main effects (*Mycorrhiza*, *P* and *Zn*). Statistically significant terms ($p \leq 0.05$) are in bold. *Myc*: mycorrhiza, *P*: phosphorus, *Zn*: zinc.

Response variable	*Myc*P*Zn*	*Myc*P*	*Myc*Zn*	*P*Zn*	*Myc*	*P*	*Zn*
Shoot dry weight	0.337	**<0.001**	0.727	**<0.001**	na	na	na
Root dry weight	0.993	**0.008**	0.683	**0.025**	na	na	na
Shoot P conc. (g kg^{-1})	0.718	**0.043**	**0.008**	**<0.001**	na	na	na
Shoot Zn conc. (mg kg^{-1})	0.256	**0.020**	0.302	0.631	na	na	**<0.001**
AMF colonisation	-	-	-	0.210	-	**<0.001**	0.578

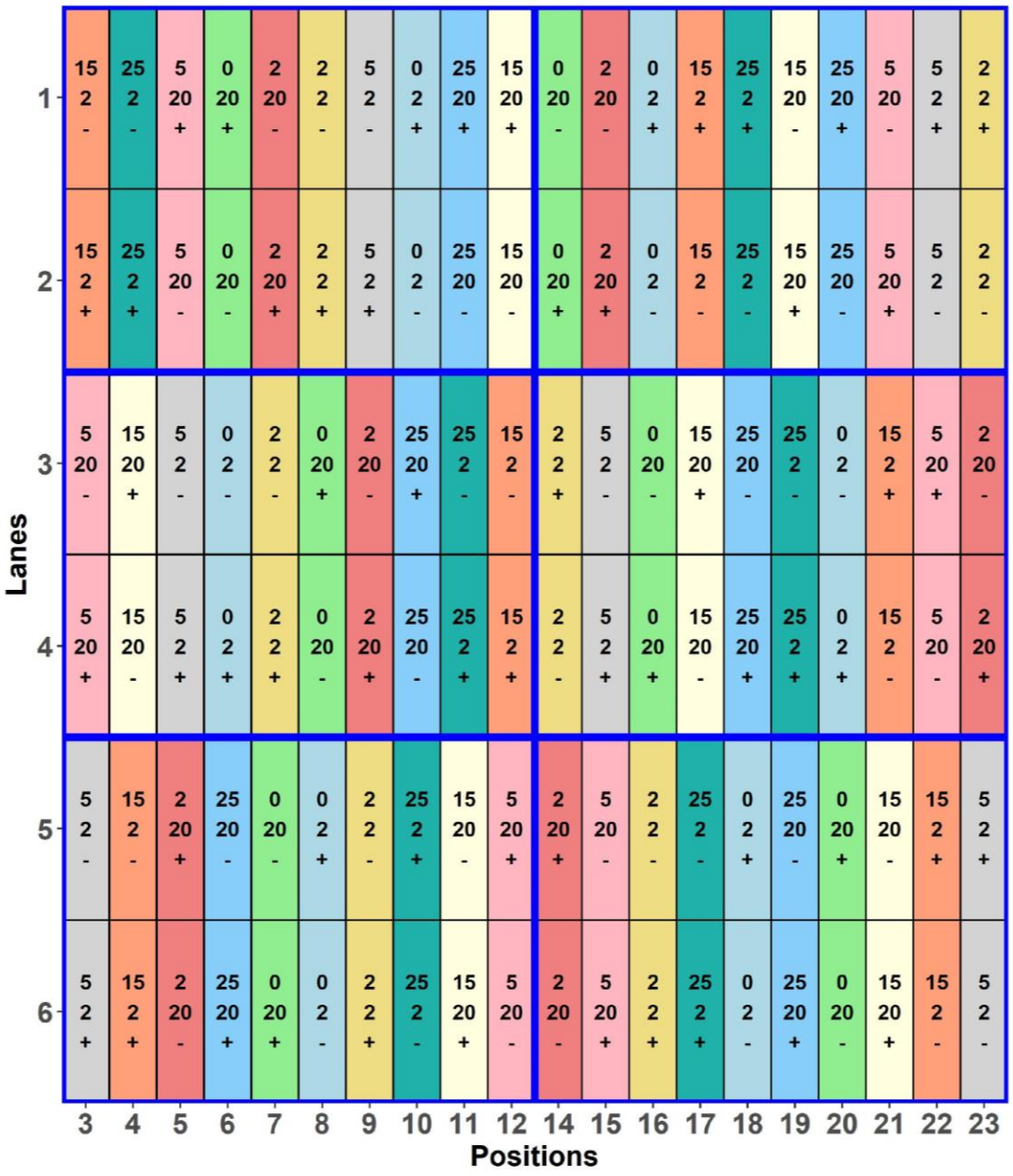

Figure S1. Diagram of the experimental design for pot placement within the Smarthouse. Pots were organised in a split-plot design, with each block of a replicate (ie., one pot of each treatment; 20 pots total) occupying 10 positions in two adjacent lanes. Within each block, the 10 nutrient treatments were randomised using part of a Latin-square design, to pairs of pots in adjacent lanes, and the two AMF treatments were then randomised within that pair. Within a segment, the first number denotes the *Zn* treatment, the second number denotes the *P* treatment, and the symbols denote whether the plant was inoculated with AMF (+) or not (-).

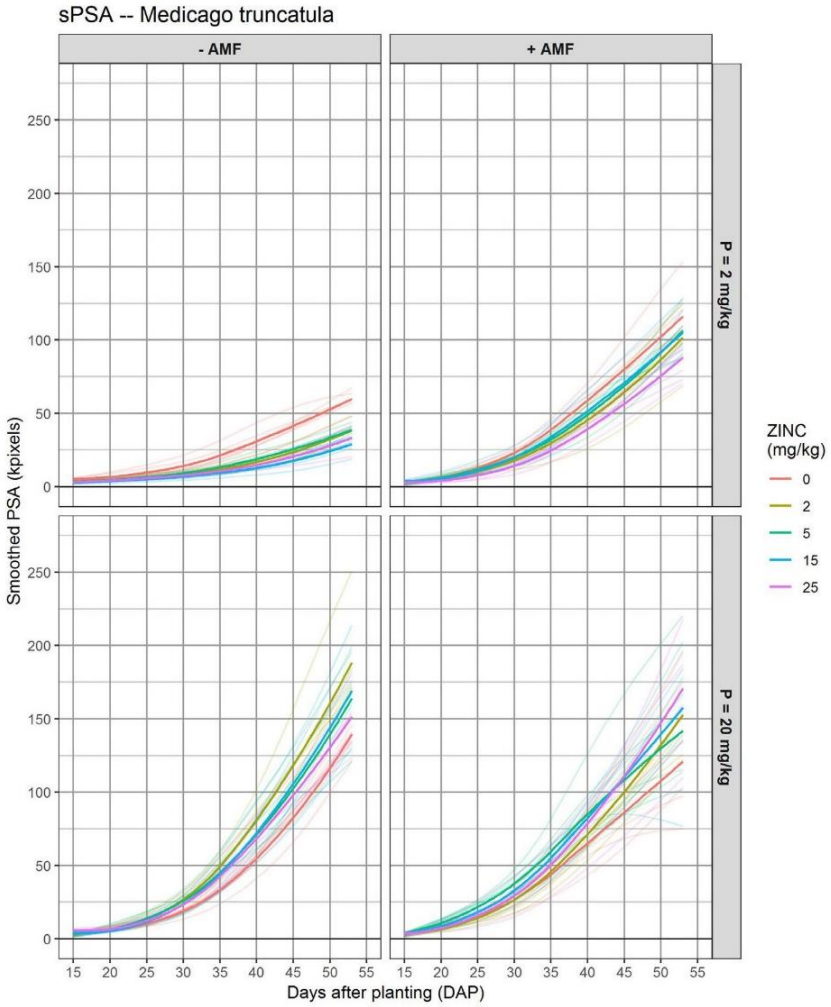

Figure S2. sPSA over time of *M. truncatula* inoculated with the AMF *R. irregularis* and grown at various soil P and Zn concentration, comprising two P levels and five Zn levels. On each panel, the darker lines represents the loess mean PSA of replicate within a treatment, while lighter lines correspond to individual replicates.

Chapter 6 – General discussion and conclusion

General discussion

In this thesis, I have focused on exploring and assessing the capacity of arbuscular mycorrhizas (AM) to improve food security, specifically, the growth and nutritional properties of a range of crop plant species and one pasture species, including their edible parts. The four experiments contained herein were designed to answer four main questions:

(i) How do different agriculturally important plant species from contrasting functional groups respond to inoculation with a single arbuscular mycorrhizal fungus species?;

(ii) To what extent does arbuscular mycorrhizal fungal inoculation and soil zinc (Zn) fertilisation impact the yield and nutritional properties of durum wheat?;

(iii) To what extent does arbuscular mycorrhizal fungal inoculation affect phytic acid (PA) concentration and bioavailability of Zn and iron (Fe) in different durum wheat genotypes?; and

(iv) How does arbuscular mycorrhizal fungal inoculation and phosphorus (P) and Zn fertilisation affect plant growth response temporally?

In this final chapter of my thesis I discuss the results of these four experiments in the broader context of food security. I also identify a number of emergent patterns in the results across the experiments. The chapter then concludes with some suggestions of possible future research that might build upon my research.

Plant identity is a strong driver of arbuscular mycorrhizal responses

In this thesis, I present results of a study in which I explored the importance of plant identity to the plant response to the formation of AM at two levels: the plant species level in *Chapter two*, and the plant genotype level in *Chapter four*.

In *Chapter two*, I present results of an experiment in which I inoculated 15 plant species with a single species of arbuscular mycorrhizal fungi (AMF). The plant species included those from contrasting functional groups such as monocots, dicots, C_3, C_4, N-fixing and non N-fixing plant species. Impacts on root mycorrhizal colonisation, growth

and nutrition of the different plant species were quantified. It was found that plant identity (species), rather than mycorrhizal status (i.e. colonised by the arbuscular mycorrhizal fungus or not), was the strongest driver of mycorrhizal colonisation, plant growth and mineral nutrition, especially the plant ionome. The variation in plant growth response to AM was reported previously in different crops (Plenchette et al. 1983) and non-agricultural plants (Klironomos 2003; Wilson and Hartnett 1998). It has been explained that plants differ in their root morphological traits and exudates which is important for the formation and development of AM (Hoeksema et al. 2010; Klironomos 2003; Wilson and Hartnett 1998). Moreover, it has been suggested that plants from the same functional group may cluster together in their responses to AM (Reinhart et al. 2012); however that was not the case here. However, among the cereals, the C_3 and C_4 plants performed differently in terms of their response to forming AM. In particular, while bread wheat, barley and durum wheat showed neutral growth response to AM, sorghum and maize (C_4) showed a positive growth response. While this finding is interesting, it needs to be further confirmed with a larger suite of plant species, before generalisations about the responses of these crop species and plant functional groups can be made. Similarly, this experiment used only one species of AMF, in one soil, at one rate of nutrient input (see *Chapter three* below) and at one time point (see *Chapter five* below); thus, generalisations should be avoided, or very carefully qualified. It would also be important to consider diverse genotypes within a single plant species (as was done for durum wheat, see below), and over the whole development cycle of the plan (e.g. using high throughput phenotyping; again, see below).

To further explore the issue of plant identity in AM responses, in *Chapter four*, I undertook an experiment in which ten genotypes of durum wheat were inoculated with a single species of AMF. Impacts on plant yield and nutrient quality traits such as protein content and Zn and Fe bioavailability were estimated. The diverse response (in terms of mycorrhizal colonisation, biomass and nutrition) of various genotypes of durum wheat to inoculation with a single species of arbuscular mycorrhizal fungus here was consistent with many other plant species such as bread wheat (Singh et al. 2012), maize (Chu et al. 2013), and faba bean (Abu et al. 2019). Therefore, in order to successfully integrate AMF into management plants of agricultural systems, a greater understanding of how plant identity and AMF affect crop performance is needed. Moreover, there is a need for such work to be conducted in a range of soils and management systems. Although this can be

addressed to some extent through greenhouse experiments, where we can easily set up a non-mycorrhizal control to compare with mycorrhizal treatments, doing so in the field is far more complex. For example, in situations where AMF may not be present or functional, in order to help farmers to make decision about whether applying AMF to a specific plants and field conditions, field-based experiments will be very valuable in confirming the greenhouse condition findings. However, it is important to note that results from field trials can differ from those obtained in the greenhouse (e.g. due to the wider soil microbiome, soil characteristics, and climate stressors) and so caution must be taken (Abu et al. 2019; Ryan et al. 2019). In *Chapter four*, I also found that no single plant genotype responded in a superior way consistently to AM in all analysed properties. However, some genotypes had greater responses to AM in term of yields and nutrition than others. These genotypes could potentially be used as material in breeding program for a better genotype associating with AMF toward high production and better nutrient content in a low input and sub-optimal conditions.

Taken together, the results of *Chapters two* and *four* indicate that plant identity, both at the species and genotype level, are strong drivers of mycorrhizal responsiveness. Therefore, choosing a genotype with superior performance when in association with AMF may bring great benefit in agricultural systems.

Zn nutrition of plants was affected by both arbuscular mycorrhiza formation and the addition of Zn and P

The uptake of Zn by plants, and Zn bioavailability in edible part of crops, have been the topic of great interest because of the abundance and severity of Zn malnutrition in people worldwide. The accumulation and distribution of Zn in mycorrhizal plants is in part the results of complex interaction between soil P and Zn. However, the understanding of this interaction, in the context of AM, is still limited (Xie et al. 2019). In this thesis, the impacts of soil P, Zn fertilisation and arbuscular mycorrhizal fungal inoculation on plant Zn nutrition were therefore explored in *Chapters three*, *four* and *five.*

Soil P is well-known as a strong driver of plants responses to arbuscular mycorrhizal fungal inoculation in root colonisation, growth and P uptake. It also has strong impact of on plant mineral nutrition, particularly Zn. This role of P addition was explored in *Chapter four* and *Chapter five* of this thesis. Two contrasting P levels were used to analyse the impact of P to mycorrhizal response of Zn in durum wheat grain

(*Chapter four*) and *Medicago truncatula* (Medicago) shoot (*Chapter five*). Phosphorus addition caused Zn reduction in durum wheat grain and Medicago shoot by arbuscular mycorrhizal fungal inoculation. This finding is consistent with previous studies in both greenhouse (Nguyen et al. 2019; Watts-Williams and Cavagnaro 2012) and field conditions (Ryan et al. 2008; Zhang et al. 2012). The increase of P availability in soil can lead to an increase in plant biomass, and thence, a reduced concentration of plant Zn and other mineral nutrients by a process of 'tissue dilution', especially in low Zn soil (Broadley et al. 2007). Phosphorus addition may also suppress the formation of AM and the activity of mycorrhizal uptake pathway, which in turn can impact the uptake and accumulation of Zn in plants (Zhang et al. 2017).

In the case of soil Zn, variable impacts of Zn fertilisation on plant Zn nutrition were found in *Chapter three* and *Chapter five* of this thesis. Firstly, while Zn fertilizers increase the concentration of Zn in durum wheat grain (*Chapter three*), Zn addition did not change Zn concentration in Medicago shoots (in *Chapter five*). Furthermore, in durum wheat when soil Zn concentration increased, grain Zn in mycorrhizal plants decreased. This was also reported previously in red clover (Chen et al. 2003), lettuce (Konieczny and Kowalska 2017) and tomato (Watts-Williams and Cavagnaro 2012; Watts-Williams et al. 2013) as the protective effect of AM in high Zn soil.

Arbuscular mycorrhizas have been widely reported as having an enhancing effect on plant Zn nutrition (Ercoli et al. 2017; Lehmann et al. 2014; Watts-Williams et al. 2015). This impact was analysed in detail in all of the experiments presented in this thesis; i.e. across a diversity of plant species and genotypes, soil conditions and plant tissues (e.g. plants shoot and durum wheat grain). The work presented in *Chapter two* revealed the great variation of Zn response to AM in different plants species which ranged from negative (e.g. sorghum and Medicago) to neutral (e.g. barley, tomato, soybean) and positive (maize and leek). Moreover, the impacts of forming AM on plant Zn nutrition differed considerably, even in the same plant species/genotype. For example, in a single genotype of durum wheat (*Chapter three*) the formation of AM did not affect grain Zn concentrations, whereas in the same genotype of durum (*Chapter four*), significant improvements in grain Zn was found. Further complicating the matter, the impact of AM on Zn was strongly affected by soil P. Similarly, for Medicago AM reduced Zn concentration in *Chapter two*, yet in *Chapter five* it increased plant Zn in high soil P and had no effect in low P soil.

Taken together, the results presented in *Chapter two, three, four* and *five* of this thesis suggest that while P addition has strong effect on plant Zn, soil Zn addition may also affect the plant Zn, but to a lesser extent. Arbuscular mycorrhizas can enhance the uptake and accumulation of Zn in edible parts (e.g. grain). However, this function depends on the context, for example: plant identity, and soil P and Zn levels.

Arbuscular mycorrhizal fungi play an important role in Zn and Fe bioavaliability in durum wheat grain

Arbuscular mycorrhizas are known to have a potential role in crop biofortification, especially for essential nutrients such as Zn and Fe (Singh et al. 2017; Upadhayay et al. 2019). This role has been reported on in many studies including a meta-analysis, that AM formation increased Zn and/or Fe concentrations in the edible parts, including cereal grain (Ercoli et al. 2017; Lehmann et al. 2014; Pellegrino et al. 2015). A similar response was found in the work presented in *Chapters three* and *four* of this thesis. The novelty of my research is that the role of AM was further considered from a human nutritional quality aspect, namely the bioavailability of micronutrients. These available concentrations of Zn and Fe for human absorption are affected by PA, the major form of P in plant seeds especially in cereal grain, the important anti-nutrient factors for most mineral nutrients, especially Zn and Fe. The bioavailability of Zn and Fe was estimated through the molar ratio of PA to mineral, the lower the ratio was the more bioavailable the nutrient was estimated to be. However, it is important to note that the molar ratios of PA to mineral used here is just a proxy to assess the bioavailability of Zn and Fe. Therefore, the bioavailability of Zn and Fe is determined by both the grain Zn and Fe concentration and the PA concentration. In *Chapter three*, the results showed that AM increased PA concentration in durum wheat grain even when grain Zn and Fe concentration was unchanged. This led to the reduction in the estimated bioavailability of Zn and Fe. In contrast, in *Chapter four*, AM not only significantly increased grain Zn and Fe concentrations across different genotypes, but also increased the concentration of PA in grain but to a lesser extent. As a result, AM increased the estimated Zn and Fe bioavailability. These contrasting results illustrate the complexity of the impact of arbuscular mycorrhizal fungal inoculation on PA accumulation in grain, which was influenced by many factors including crop species and genotype, as well as the availability of nutrients in the soil including P, Zn and Fe. Besides arbuscular mycorrhizal fungal inoculation, soil P addition significantly increased grain PA in durum wheat, and

thus, significantly decreased the estimated Zn and Fe bioavailability. Taken together, the potential of AM in biofortification was significant in our research in agreements with previous studies. Moreover, my studies emphasized the need to determine the effect of AM on PA in cereals grain in order to assess the nutritional value of AM on the bioavailability of these essential minerals for human consumption.

The impact of arbuscular mycorhizas on plant growth changes temporally

The work presented in *Chapters two, three* and *four* focused on a final destructive harvest; that is, a single point in time. However, it is well known that plant responses to various conditions change over the chronological and ontogenetic development of the plant (Miller et al. 2014). To further explore this matter, a high-throughput shoot phenotyping platform was used to demonstrate that the effect of AM on the growth Medicago changes over the life of the plant (*Chapter 5*). In particular, AM can still positively affect the growth of Medicago plants even in high soil P condition which was not apparent in single point harvest data. Therefore, it suggested that the neutral mycorrhizal growth responses found in Medicago plants in *Chapter two* result, of the harvest data may not represent the true effect of AM on the plant growth but the accumulated effect at that time point. Moreover, choosing harvesting time is essential and may affect the conclusion when analysing the effect of AM on plant growth. On the other hand, in the study of Watts-Williams et al. (2019) at high soil P, the growth response of Medicago to AM was unchanged over the growing course. This, again, highlights the context dependence of mycorrhizal effects, particularly on the soil nutrient status (e.g. P and Zn). In order to further elucidate this matter, it would be useful to look at the temporal growth responses to AM of agriculturally important crops, to explore the impact of AM on the plants above-ground development. It is likely that similar patterns to Medicago may be seen in crops which are highly responsive to arbuscular mycorrhizal fungal inoculation in growth in low P, and neutrally respond to AMF in high P. It would be more interesting when analysing different genotypes with diverse response pattern to AM of the same plant species. Moreover, it would be very informative to look at the impact of AM on plant nutrition over time, and non-destructively in parallel with plant growth. High throughput analysis of plant mineral nutrition, particular P and Zn, can be achievable using a hyperspectral imaging system (Liu et al. 2015; Pandey et al. 2017), and would be extremely useful in efforts seeking to further explore the issues raised in this thesis.

The limitations of this body of work

Firstly, only a single arbuscular mycorrhizal species, *Rhizophagus irregularis*, was used to analyse the impact of AM on plants` growth and nutrition in greenhouse condition in all of the experiments presented here (*Chapter two*, *three*, *four* and *five*). It is acknowledged that there are great number of native AMF species existing in soil even in agricultural systems has suboptimal condition for AM (Zubek et al. 2013). Therefore, the interaction/competition of AMF community and the impact of arbuscular mycorrhizal succession pattern (Chagnon et al. 2013) which may contribute to the acquisition of various minerals (e.g. P, N, Zn and Fe) throughout the life cycle of the crops was not included in my studies. Secondly, the size of pot used in our greenhouse experiment was also rather small which may cause variation of arbuscular mycorrhizal fungal performance to real field soil because of the difference in the spatial distribution of extraradical hyphae of AMF. This could be not only a limiting factor for the growth but also bias/change nutrient balance of the plants; e.g., balance among biomass, P, phytic acid, and Zn/Fe via restricting root growth.

Therefore, in order to estimate the impact of AMF in the agricultural context, it is important to continue to investigate the impact of AM in the field conditions. The results of my studies will be helpful when planning future field experiments.

Conclusions

The impact of AM on plant growth and nutrition of agriculturally important crops are highly variable. In fact, in the study presented in this thesis, neutral and negative effects were found more often than positive effects. However, AMF still have a role to play in sustainable agricultural systems, especially in the context of the fossil P is running low, the fertiliser cost is increasing. That is, arbuscular mycorrhizal fungal inoculation can compensate the loss that reducing inputs may cause in a sustainable manner. Moreover, the results of my research suggest that in order to apply AMF in agriculture practice for food security purpose, there are many factors that should be considered, as follows:

Firstly, the crop identities including: species and genotypes can cause great variation in plant mycorrhizal responsiveness which is unpredictable. Therefore, it is important to pay attention to the specific plant species and genotypes in certain environmental conditions for expected outcomes. Moreover, breeding genotypes with

superior responses to AMF in yield and quality in the poor nutrient soil status is also suggested.

Secondly in agricultural practice, P application can strongly hide or change the effects of AM. In particular, increased soil P changed the positive the effect of AM on grain P, Zn and Fe to neutral and negative in some durum wheat genotypes.

Thirdly, in order to assess the effect of AM on the bioavailability of Zn and Fe in cereal crops, it is important to examine the effect of AM on PA concentration of the grain. Because AM can not only affect the concentration of these mineral nutrients but also the concentration of PA. More importantly, PA is also a determinant of the bioavailability.

Fourthly, the effect of AM on the bioavailability of Zn and Fe in cereal crops is variable and context-dependent. Because the formation of AM can impact both determinants of the bioavailability of Zn and Fe, and it can be influenced by the interaction between plant identity, the presence or absence of AMF, and soil nutrient status (e.g. P, Zn). Moreover, soil P, can directly and strongly affect PA concentration.

Lastly, the response of plants to AMF can change over the life time of the host plants. Therefore, assessing impacts of AMF on plant growth over time will improve our understanding of the impact of forming AM on plants.

Future directions

In undertaking the work described in this thesis, a number of potential future research directions were identified. These include:

Firstly, a broader understanding of the impact of different mycorrhizal isolates on different plant varieties to boost the bioavailability of mineral nutrient accumulation across major crops and environment conditions. This knowledge can be obtained from greenhouse experiments using homogenous sieved and sterilised soil, in pots, with frequently watering and controlled conditions. In particular, it is important to explore the impact of AM on PA concentration and the bioavailability of Zn and Fe in grain in other commercially important genotypes of cereal species, such as sorghum or maize. These two plant species are known to generally have positive growth responses when inoculated with AMF. In addition, the impact of soil condition including soil nutrients (e. g. P, N, Zn) availability and forms (e.g. organic/inorganic) also need to be counted; or environmental stress factors such as temperature difference between day and night, water. In doing so, the true effect of AM on the human nutritional quality of crops will be revealed.

Secondly, it will also be important to find out if there are any indicators that can be used to predict the outcomes of AMF impacts on plants over their entire growth cycle. It will be useful to determine how AM and P fertiliser affect the growth overtime of various genotypes of the same crop species, which differ in their growth responses. This can be done using high-throughput phenotyping systems to measure plant growth over time. This will enhance the understanding of interaction between plant and AM and the impact of AM on plant`s development.

Moreover, the understanding gained from greenhouse experiments is then needed to be verified under field conditions in different agricultural systems. A reason that may cause the differences of the field and greenhouse outcomes is the native soil biota. Therefore, in order to estimate the potential AM in agricultural practice, it is important to analyse the impact of native AM fungal population in soil of important agricultural regions to crops nutritional quality, for example on PA and the bioavailability of Zn and Fe in cereals grain. After that, in order assess the impact of AM in agricultural systems, it would be useful to extend the work undertaken here under field conditions.

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