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# Optimising phosphorus supply to plants by combining inorganic P fertilisers and organic amendments, and the roles of arbuscular mycorrhizas

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# THESIS ABSTRACT

Inorganic phosphorus (P) fertilisers are used widely to maximise crop yields. However, in many cases, much of the P fertiliser applied is unavailable to plants due to P adsorption. Consequently, excess inorganic P fertiliser application often ensues. The consumption of inorganic P fertiliser has increased over the last five decades causing increasing concern around the availability of mineral P resources, and growing interest in identifying alternative sources of P to support sustainable agricultural production. Inorganic P fertilisers, organic P-rich materials and arbuscular mycorrhizal (AM) fungi are valued for their abilities to supply P to plants. It may be possible to integrate these three elements to optimise plant P nutrition. To this end, the research presented in this thesis worked to understand how plants respond to the combined use of inorganic P and organic amendments as fertilisers, and further, how they interact with the AM symbiosis.

Across four independent experiments, I examined the growth and nutritional responses of 76R (mycorrhizal) and *rmc* (non-mycorrhizal) tomato plants to different P sources (inorganic, organic and mixed). In the first experiment, I used inorganic P fertiliser alone, organic P-rich material alone, or combinations of the two P sources, at a low P application rate. The second experiment was developed based on the findings of the first experiment, in which three P fertiliser sources were tested at low, medium and high application rates. The third experiment was established where low and high nitrogen (N) application rates were investigated together with low and high P application rates. In addition, shoot growth responses of the plants were examined over time using high throughput phenotyping. Finally, I formulated six P fertilisers by co-granulating different ratios of MAP/chicken litter. The N/P ratios of the fertilisers were balanced by adding urea into the formulations. A MAP only control (no additional urea) was also included. Physical and chemical characteristics of formulations were measured along with the kinetics of $NH_4^+$-N and P release from the fertilisers, before testing their impacts on plant growth, nutrition and arbuscular mycorrhizal responses.

Overall, while the inorganic P source alone led to faster early shoot growth, the later shoot growth was overtaken by the combined P sources. The combined P sources increased plant growth, which was explained by gradual P release that was more closely aligned to plant P demand over the growth cycle. Thus, the combined use of inorganic P and organic P-rich material was a suitable method to supply P to plants. Furthermore, my research also highlighted an important knowledge gap on the interacting effects of AM fungi with different P source materials, which may help improve P use efficiency and crop yields. I also successfully produced combined P source fertilisers that differed in their nutrient release properties and impacts on plants.

# THESIS 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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Hue Ngo

25/06/2021

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| AM | Arbuscular mycorrhizal |
| AMF | Arbuscular mycorrhizal fungi |
| DAP | Day after planting |
| HTP | High-throughput shoot phenotyping |
| ICP-OES | Inductively coupled plasma atomic emission spectroscopy |
| MAP | Mono ammonium phosphate |
| MGR | Mycorrhizal growth response |
| MNP | Mycorrhizal nitrogen response |
| MPR | Mycorrhizal phosphorus response |
| N | Nitrogen |
| P | Phosphorus |
| $P_i$ | Inorganic phosphorus |
| $P_o$ | Organic phosphorus |
| PSA | Projected shoot area |
| *rmc* | Tomato genotype unable to form association with the AMF isolate *R. irregularis* WFVAM10 |
| SE | Standard error of the mean |
| SEM | Scanning Electron Microscope |
| sPSA | Smoothed projected shoot area |
| sPSA AGR | Smoothed absolute growth rate |
| sPSA RGR | Smoothed relative growth rate |
| 0:4 | P fertiliser formulation, 0% P from inorganic P source: 100% P from organic P source |
| 1:3 | P fertiliser formulation, 25% P from inorganic P source: 75% P from organic P source |
| 2:2 | P fertiliser formulation, 50% P from inorganic P source: 50% P from organic P source |
| 3:1 | P fertiliser formulation, 75% P from inorganic P source: 25% P from organic P source |
| 4:0 | P fertiliser formulation, 100% P from inorganic P source: 0% P from organic P source |
| 76R | Tomato genotype able to form association with the AMF isolate *R. irregularis* WFVAM10 |

# CHAPTER 1: INTRODUCTION

## 1. Thesis outline and approach

This thesis has been prepared in accordance with the University of Adelaide's guidelines for 'Thesis by publication'. Chapter 1 presents the introduction to my thesis, starting with the outline and approach taken. Following that, I present a general literature review, which leads to the overall aims and objectives of my research. This is concluded with a summary of the outcomes of my PhD program.

The following experimental/data chapters (Chapter 2-5) are presented as a standalone paper (published, in review, or in preparation). Each of these chapters contain their own detailed introduction, thus, the introductory chapter of the thesis is kept deliberately short to avoid repetition. The final chapter of my thesis is a general discussion, in which I integrate the findings of the individual experiments, leading to my overall conclusions. This final chapter also identifies potential directions for future work on this topic.

N.B. The published papers is presented in the format that it was published in, with additional page numbers to maintain continuous pagination throughout the thesis. Unpublished manuscripts are presented in a format similar to that of a published paper, with Tables and Figures inserted throughout the chapters.

## 2. Background

### *2.1. Requirement for optimising phosphorus supply to crops*

Phosphorus (P) is one of the primary macronutrients in plants, and accounts for 0.3- 0.5 % of total plant dry weight (Hawkesford *et al.*, 2012). Phosphorus is a structural component of nucleic acid and phospholipids. Phosphorus also acts in every plant metabolic process, as it is part of the energy transfer compound adenosine 5'-triphosphate (ATP). Phosphorus is also involved in regulating various plant environmental feedback responses, such as protein phosphorylation (Xu *et al.*, 2019). Thus, phosphorus is an essential macronutrient for plant growth and development (Hawkesford *et al.*, 2012; Jones, 2011).

In agricultural systems, many soils have insufficient amounts of P to meet crop demand (Reijnders, 2014). For example, the level of available soil P is categorised as low to very low in a third of cropland in European Union (Tóth *et al.*, 2014). Moreover, following P fertiliser application, there are significant increases in crop yields in both tropical (40%) and temperate regions ( 100%) (Ros *et al.*, 2020), indicating a need for applying external P sources to maximise crop yields (Whitcomb *et al.*, 2014; Zhang *et al.*, 2017). It is estimated that P inputs in agricultural soils will need to increase by 51 - 86% by the year 2050, if we are to meet the food demands of our expanding population (Mogollón *et al.*, 2018). However, the dominant sources of mineral P fertilisers are derived from finite terrestrial and ocean phosphate rock deposits (Ashley *et al.*, 2011). While there is ongoing discussion regarding the timeline of mineral P reserve depletion, the quantity and quality of readily accessible P resources is

decreasing globally (Koppelaar and Weikard, 2013). Thus, there is a need to use P resources as efficiently as possible.

The ongoing and intensive application of P fertilisers to agricultural soils has raised some concerns (Bouwman *et al.*, 2017). Phosphorus fertiliser use efficiency is often quite low, with an average of 15- 30% of P fertiliser applied being taken up by plants (Dhillon *et al.*, 2017; White, 2009). This low P use efficiency can lead to continued P inputs, which are both costly to farmers, and can lead to the accumulation of (unavailable) P residues in the soil over time (McLaughlin *et al.*, 2011; Roberts and Johnston, 2015). Excess P can reach water bodies via leaching (as precipitates or bound to soil particles or organic matter), surface run off or via erosion, potentially leading to algal blooms, hypoxia and eutrophication (Daloğlu *et al.*, 2012; Fink *et al.*, 2016; Garnache *et al.*, 2016; Khan *et al.*, 2018; Scavia *et al.*, 2014). Furthermore, the long-term application of P fertilizers may be an unsustainable agricultural practice; the application of solely inorganic P to soils may lead to a reduction in soil organic carbon, which can decrease soil biological activity, affect nutrient mineralisation and hence further constrain crop yields (Velásquez *et al.*, 2016). Taken together, P is a finite resource, that when applied to soils is often used inefficiently, which can affect farmer's profits and have negative environmental consequences. Thus, there is a need to find alternative P sources, while also optimising P supply to plants for sustainable agricultural production.

### *2.2. Phosphorus behaviour in soils affects plant phosphorus acquisition*

Phosphorus in soils comprises inorganic and organic forms that are allocated to three common P pools (Nesme *et al.*, 2014) (Fig. 1). The fixed P pool contains P in inorganic forms that are practically irreversibly bound to soil particles and recalcitrant organic P forms. The active P pool includes readily exchangeable inorganic P and easily mineralized organic P forms. The solution P pool contains labile inorganic P and low molecular weight organic P substances that are available sources for plants (Butusov and Jernelöv, 2013; Roberts and Johnston, 2015; Shen *et al.*, 2011). In soils, while the solution P pool can be equilibrated with the active P pool, the rate of P desorption to solution is slower than P adsorption by surfaces of clay minerals (soil reactive sites) (Bolland *et al.*, 2003; Smeck, 1985). Thus, plants may perform below their potential yield due to low available P, even when the total P in soil is sufficient (Schachtman *et al.*, 1998). Accordingly, in many agricultural systems, maintaining P in soil solution plays a significant role in determining crop yields (Lambers *et al.*, 2006; Lopez-Arredondo *et al.*, 2014).

In terms of mobility, the movement of P in soil toward the roots by diffusion is extremely slow, also the concentration of P in soil solution is generally lower compared to in plant cells (e.g. 10 µM vs 100 mM) (Hawkesford *et al.*, 2012; Holford, 1997). Thus, (direct) plant P uptake is an energy intensive process that involves $H^+$–ATPase co-transport (Ham *et al.*, 2017). Furthermore, the mobility of P in soil

is limited due to its negative charges attracted by positive charges of soil clay mineral (Clarkson, 1981). Thus, the movement of P in soil solutions toward roots via diffusion and mass flow is of low consequence to plant P acquisition (Asomaning, 2020). To overcome these challenges, plants have evolved a series of traits that can help enhance and optimise plant P acquisition.

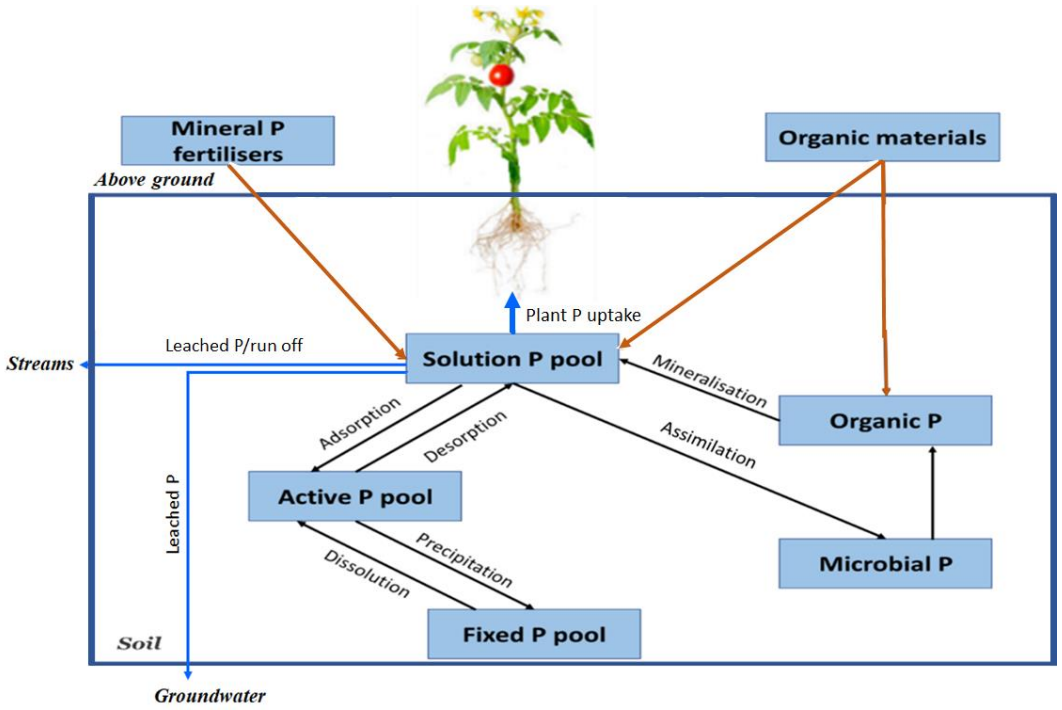


Fig. 1. The dynamic pools of phosphorus in soil that affect plant P uptake (Adapted from Nesme *et al.* (2014)). This simplified diagram omits some chemical processes and does not represent flux rates or pool sizes.

## *2.3. Plant phosphorus acquisition strategies*

The active transport of P via Pi transporters is a critical mode determining the yields of P uptake in plants (Richardson *et al.*, 2011; Roberts and Johnston, 2015). Plant Pi transporters are located on the root epidermis and root hairs. Furthermore, plants have evolved various strategies to optimise active P acquisition. For example, plants improve root P scavenging by enhancing root proliferation in soil P hotspots, increasing the frequency and length of root hairs, forming root clusters and modifying root architecture towards more efficient P uptake (Funayama-Noguchi *et al.*, 2015; Holford, 1997; Lambers *et al.*, 2008). Plants also indirectly modulate available P in the soil solution via exudation of organic compounds such as carboxylates, phenolics and phosphatase. For example, carboxylates displace Pi or Po in soil metal cation complex, following by Po hydrolysis by phosphatase, hence releasing Pi which is in the form available to plants (Lambers *et al.*, 2006).

Plants can also obtain P from soil by forming associations with arbuscular mycorrhizal (AM) fungi, a group of fungi belonging to the phylum Glomeromycota (Krüger *et al.*, 2012; Smith and Read, 2008; Stürmer, 2012). AM fungi are common in most all soil types and can form a mutualistic symbiosis

with the roots of the vast majority of terrestrial plant species (Willis *et al.*, 2012). The fungi transport Pi into plants via the mycorrhizal pathway (Fig. 2) (Smith, 2003). External AM hyphae contain high-affinity Pi transporters that allow active P uptake (Javot *et al.*, 2007; Santner *et al.*, 2012). Internal AM hyphae grow between and within root cortical cells (Cavagnaro *et al.*, 2001). Within the cortical cells the hyphae form highly branched structures, called arbuscules, which are the site of plant-fungal nutrient exchange (Smith and Read, 2008). The interface between the arbuscules and the plant plasma membrane is named the peri-arbuscular space (i.e. between the plant and fungal membranes). The fungi unload P into the peri-arbuscular space, and plants then actively take up the P into their cytosol (Bonfante and Genre, 2010; Garcia *et al.*, 2016; Javot *et al.*, 2007; Karandashov and Bucher, 2005; Yang and Paszkowski, 2011). This P can then be used by the plant for its day-to-day activities. It has been demonstrated that up to 100% of a plants P supply can occur via the mycorrhizal pathway (Schnepf *et al.*, 2008; Smith, 2003).

Outside the root, AM fungi grow an extensive mycelial network in soil, that allows the fungi to scavenge for P beyond the P depletion zones than form around roots (Smith and Smith, 1990). The external hyphae of AMF have been found to extend more than 30 mm beyond the surface of the roots they are colonising (Mai *et al.*, 2019). Moreover, the hyphae of AMF can access small soil pores that are inaccessible to roots. Once hyphae take up P form the soil, it is transported into a fungal vacuole where poly-P is formed for long distance transport into the plant roots; this process inside AM hyphae can overcome the slow diffusion rate of P from the soil solution to root surfaces (Ezawa *et al.*, 2002; Hijikata *et al.*, 2010; Ohtomo and Saito, 2005). Therefore, it has been suggested that the AM nutrient uptake pathway is an extra benefit of the host plants for P acquisition, especially in P limiting environments (Bucher *et al.*, 2014; Giovannetti *et al.*, 2017; Smith *et al.*, 2011; Watts-Williams *et al.*, 2015).

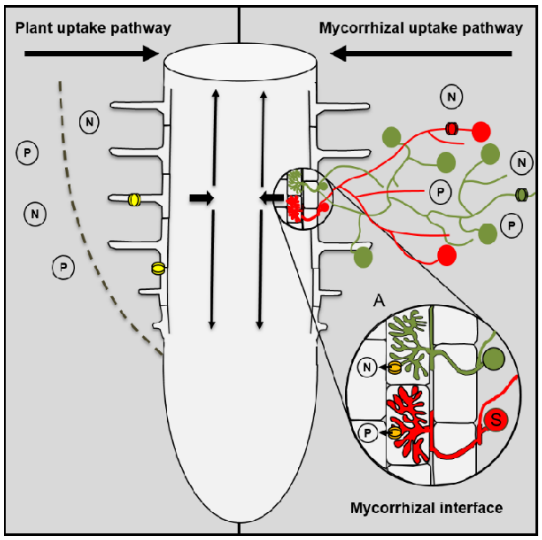


Fig. 2. Schematic illustration of the plant (direct) nutrient uptake pathway and arbuscular mycorrhizal nutrient uptake pathway (Bücking and Kafle, 2015)

### *2.4. Mismatch in phosphorus supply vs plant phosphorus uptake*

As noted above, much of the P applied to soil is in inorganic forms that are rendered unavailable to plants (see Fig. 1), and so excess P fertilisation often ensues. This is largely because P fertiliser is added to the soil at the start of the growing season, but plant demand occurs over the entire plant growth cycle. Thus, in order to supply plants with enough P to support their entire development, excess P is applied. Inorganic P fertilisers sourced from phosphate rock, which make up a majority of fertiliser usage in agriculture (Lu and Tian, 2017; Roberts and Johnston, 2015), suffer from this problem. Ideally, a P fertiliser would have slow release properties, which would allow soil P supply to match that of the plant over its development. Organic P-containing materials that must be mineralised into plant-available forms may present one option to achieve this goal.

Whereas the mineral P reserve is finite, P-rich organic materials such as animal manures – a waste product of the livestock industry- are increasingly available. Although, such waste products pose challenges in term of their disposal, they may also represent an opportunity to supply P (and other nutrients and carbon) to agricultural soils (Hossain *et al.*, 2017; Pagliari and Laboski, 2012; Rothwell *et al.*, 2020; Senthilkumar *et al.*, 2014). While the use of animal waste as a source of nutrients in agriculture is by no means a new concept, if we are to make use of livestock manures as P fertilisers, appropriate technological and strategic management will be required (Bateman *et al.*, 2011). Moreover, if they are to be used as a predictable source of nutrients, we need a good understanding of their behaviour (in particular nutrient release) in soil. Unlike inorganic P fertilisers, P-rich organic materials are heterogeneous P sources, comprising a mixture of inorganic and organic P forms. For example, research showed that chicken litters contain 15 % of their dry weight as P, of which 65 % are directly available to plants (Mackay *et al.*, 2017b). All remaining P needs to be mineralised before it can be taken up by plants.

As noted above, while P fertiliser is applied to maximise plant yields, most of the P applied remain in the soil, resulting in low P fertiliser use efficiency (i.e., P applied vs P taken up by the plant) (Withers *et al.*, 2014). Possible reasons for increasing soil P residues are fertiliser-derived P becoming immobile and unavailable to plant due to adsorption, precipitation and microbial assimilation (see Fig. 1) (Vitousek *et al.*, 2010). Phosphorus immobilisation may be favoured when common inorganic fertilisers are applied to soil due to their fast rate of P release into the soil solution (Holford, 1997), and the formation of strong P sorption reactions as a result of high P concentrations around fertiliser granules (Asomaning, 2020). In contrast, the application of organic materials can help to increase the efficiency of P fertilizers by enhancing P solubility (Song *et al.*, 2007), and reducing P fixation (Li *et al.*, 2012). One drawback of using such materials as a fertiliser is that they can have a low nutrient content per dry weight, leading to high transport costs (Fealy and Schröder, 2008). Furthermore, compared to inorganic P fertilisers, organic materials can be hard to pelletise, which may limit the application of

organic materials as fertilisers in the field (Stelte *et al.*, 2012). Thus, further research is needed to understand the various drawbacks and advantages of inorganic vs organic P sources for improving crop P use efficiency (Withers *et al.*, 2014).

The requirement of plants for P changes over the plant's development, with critical periods where P supply is considered the most important factor for determining yield and nutrition (McDonald, 1994). There is evidence to suggest that there is little difference in the impacts of inorganic and organic fertilisers on the early stages of plants grow, as both sources contain readily available P (Xu *et al.*, 2020). However, the type of P fertiliser (i.e. inorganic versus organic) is important in latter stages of plant development, indicating a need to 'match' the rate of P release for different fertiliser materials, with plant demand (Van Noordwijk, 1990; Xu *et al.*, 2020). As noted above, in order to overcome this issue, P fertilisers often applied well in excess of plant demand at the start of growing season, to avoid a deficiency later in the growing season. In addition to the risk of excess fertiliser being leached in wet conditions, in dry conditions, P precipitation (which reduces availability) may be exacerbated (McLaughlin *et al.*, 2011; Ven *et al.*, 2019). In contrast, organic P fertilisers need to be mineralized before the P is available to plants, which can result in a slow release of P into the soil that may be better matched to plant demand (Quilty and Cattle, 2011). However, it is important to note that mineralization processes are highly dependent on environmental conditions (Dey *et al.*, 2019; Malik *et al.*, 2012). Taken together, it is clear that fertilisers with slow-release P characteristics are desirable, and that organic materials may be one option to achieve this. The reactions of P release need to be understood so that the fertilisers behave in a predictable manner, and materials also need to be able to be formulated into granules that are compatible with available machinery (Bateman *et al.*, 2011; Lewu *et al.*, 2020).

While P fertilisation is important in maximising crop yields, it can also adversely impact the formation of AM which also have a role to play in plant P acquisition (Breuillin *et al.*, 2010a; Gu *et al.*, 2011; Javot *et al.*, 2007; Nouri *et al.*, 2014; Sarabia *et al.*, 2017). Mycorrhizal colonisation is generally lower in soils with high available P (Balzergue *et al.*, 2013; Gosling *et al.*, 2013; Kobae *et al.*, 2016; Liu *et al.*, 2016; Ngo and Cavagnaro, 2018). In soil with high concentrations of available P, plant roots may reduce the excretion of strigolactone to the rhizosphere, an important hormone for initiation of AM spore germination and AM hyphal branching and metabolism (Balzergue *et al.*, 2011; Czarnecki *et al.*, 2013). High available soil P can also down-regulate AM-inducible genes, causing the inhibition of AM colonization (Breuillin *et al.*, 2010b; Nagy *et al.*, 2009). Furthermore, although it appears that better AM colonisation in roots leads to greater shoot P content, the impact of AM colonisation on plant growth is complex; positive mycorrhizal response, for instance, may not be observed in high available P soil conditions (Ji and Bever, 2016; Lekberg and Koide, 2005; Smith and Smith, 2011; Treseder, 2013). The growth suppression of mycorrhizal plants may be a consequence of overall lower total P uptake

due to a reduction in P acquisition through the direct P-uptake pathway, as phosphate starvation induced genes are down regulated in mycorrhizal roots, while P uptake via mycorrhizal pathway may not fully compensate for the direct pathway (Smith, 2003; Smith *et al.*, 2011). Thus, while P fertiliser application works to increase available soil P, the P fertiliser impact on AM association may decrease total plant P uptake despite higher available soil P. Thus, if plant P acquisition in mycorrhizal plants with low P inputs were equal to those with high P inputs, it may not be necessary to supply P fertilisers at high application rates. Given that the use of different P sources could change soil P dynamics, it is important to understand the growth response over time of mycorrhizal plants to different P sources. On way to achieve this is through the use of high-throughput phenotyping (Riley *et al.*, 2019; Watts-Williams *et al.*, 2019).

### *2.5. Closing the loop: approach to optimise phosphorus supply to plants for sustainable agricultural development*

Increased P use efficiency and recycling of P resources are important measures to ensure P security (Cordell and White, 2013). As P use efficiency depends on the ability of soils to supply adequate available P to plants, and the subsequent ability of plants to acquire available P, combining inorganic fertilizers and organic materials is an integrated method for addressing P security (Reijnders, 2014; Roberts and Johnston, 2015). This is because the inorganic P sources can immediately provide plant P, especially at the initial growth stage where root systems have not been fully developed, and the organic P sources can gradually provide P for subsequent plant growth (Akhtar *et al.*, 2011; Grant *et al.*, 2001; Moe *et al.*, 2017; Zhang *et al.*, 2017). In addition, while the application of inorganic P fertilizer tends to restrict AM formation (Jeffery *et al.*, 2017), the application of organic materials such as composts to soils is in most cases compatible with the formation of arbuscular mycorrhizas (Cavagnaro, 2015; Mackay *et al.*, 2017a). It is suggested that the application of inorganic P sources together with organic P sources may maintain sufficient available P in soils, but not at a level that retards the formation and functioning of arbuscular mycorrhizas (Mackay *et al.*, 2017a; Van Geel *et al.*, 2016). Moreover, recent advances in material technology are leading to the development of P fertilisers that have slow release characteristics (Frazão *et al.*, 2019; Kabiri *et al.*, 2020; Mazeika *et al.*, 2016; Weeks and Hettiarachchi, 2019). Therefore, the integration of inorganic fertilizers with organic materials may help reduce P waste, provide a P source that is more closely matched to plant demand, and may not adversely affect AMF. In this thesis I will, therefore, explore the recycling P-rich waste materials, with a view to developing a slow release P fertiliser that considers the role of AM in optimising phosphorus supply to plants.

## 3. Aims and objectives of the research

The overarching aim of the research presented in this thesis was to understand how plants respond to the combined use of inorganic P and organic amendments as fertilisers, and further, how they interact with the arbuscular mycorrhizal symbiosis.

The objectives were to:

- Quantify the effects of various combination ratios of an inorganic P source and an organic material on plant growth, nutrition and arbuscular mycorrhizas. (Chapter 2)
- Evaluate the effects of different P sources at low, medium and high application rates on plant growth, nutrition and arbuscular mycorrhizas. (Chapter 3)
- Investigate growth responses of plants and arbuscular mycorrhizas to different P sources over the life of the plant using high-throughput shoot phenotyping. (Chapter 4)
- Formulate and characterise the physical and chemical properties of slow-release P fertilisers made from inorganic and organic P sources, and examine their subsequent impacts on plants and arbuscular mycorrhizas. (Chapter 5)

## 4. Outcomes of PhD Program

❖ *Published paper*

Ngo, H.T.T., Watts-Williams, S.J., Cavagnaro, T.R., 2021. Mycorrhizal growth and phosphorus responses of tomato differ with source but not application rate of phosphorus fertilisers. Applied Soil Ecology 166, 104089.

❖ *Manuscript under review*

Ngo, H.T.T., Cavagnaro, T.R., Jewell, N., Brien, C.J., Berger, B., Watts-Williams, S.J., (Under review). Combining organic and inorganic sources of phosphorus fertiliser sustains tomato growth over time. Journal of Experimental Botany. Special issue: Plant Phenotyping for a Sustainable Future.

❖ *Yet unsubmitted manuscripts*

Ngo, H.T.T., Watts-Williams, S.J., Cavagnaro, T.R.,. Organic P source increased growth, but had lower arbuscular mycorrhizal P uptake benefit in tomato plant compared to inorganic P source.

Ngo, H.T.T., Watts-Williams, S.J., Panagaris, A., Baird, R., Michael J. McLaughlin, M.J., and Cavagnaro, T.R. Formulation and evaluation of chicken litter–MAP organomineral fertilisers.

❖ *Seminars*

May 2018: PhD Introductory seminar. Soil science group. The School of Agriculture, Food and Wine. The University of Adelaide.

November 2018: Major review seminar. Soil science group. The School of Agriculture, Food and Wine. The University of Adelaide.

- *Internship program*

May 2019: 2019 Postgraduate Internship Award. The Plant Accelerator, The University of Adelaide, Waite Campus, Adelaide SA.

- *3 Minute Thesis*

July 2019: "Extracting gold from chicken poo". Three-minute thesis competition. The School of Agriculture, Food and Wine. The University of Adelaide.

- *Symposium*

September 2019: "Arbuscular mycorrhizal roles in plant growth and phosphorus nutrition in different phosphorus source scenarios". Postgraduate Symposium. The School of Agriculture, Food and Wine. The University of Adelaide.

- *Soil judging competition*

October 2019: Participant. 7th Australian Soil Judging Competition. Strathalbyn, South Australia

- *Adelaide Graduate Award*

October 2020: Adelaide Graduate Award for employability skills development through extra-curricular activities. The University of Adelaide.

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# CHAPTER 2: ORGANIC P SOURCE INCREASED GROWTH, BUT HAD LOWER ARBUSCULAR MYCORRHIZAL P UPTAKE BENEFIT IN TOMATO PLANTS COMPARED TO INORGANIC P SOURCE

# Statement of Authorship

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Organic P source increased growth, but had lower arbuscular mycorrhizal P uptake benefit in tomato plants compared to inorganic P source

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## Abstract

Phosphorus (P) application is required to maintain plant yields; however, P fertiliser production makes use of finite inorganic P sources. While P-rich organic material is considered as a sustainable P resource, the combination of inorganic and organic P sources might improve the supply of P over the life of the plants. The aim of this research is to investigate the effects of soil amended with different P sources on the growth and nutrition of tomato plants and arbuscular mycorrhizas. While total P was maintained, different P sources were produced by incorporating inorganic P and organic materials in five ratios: 4:0, 3:1, 2:2, 1:3 and 0:4. Plant dry weight, nutrition and mycorrhizal responses were evaluated in tomato mycorrhizal and non-mycorrhizal plants. The results showed that the application of the organic material alone and, to a lesser extent, the combined P sources enabled plants to gain a growth and nutritional advantage. However, the benefit of AM on plant P uptake was more pronounced in the inorganic P alone source. The findings highlighted the cases for using organic material as an alternative P source and arbuscular mycorrhizal fungi to improve P supply to plants.

**Keywords:** Agricultural wastes; Arbuscular mycorrhizal symbiosis; Organic amendment; Recycling; Soil phosphorus; Tomato

## 1. Introduction

Synthetic fertilisers are used widely to maximise crop yields. In many cases, much of the fertiliser applied is unavailable to plant roots (Khan *et al.*, 2018). For example, a major proportion of phosphorus (P) fertiliser applied is fixed by the clay soil component (Rajput *et al.*, 2014). As a consequence, while highly effective, excess P fertiliser application often ensues (Khan *et al.*, 2018). As the consumption of P chemical fertiliser has increased over the last five decades (Lu and Tian, 2017), there is increasing concern around the availability of easily accessible mineral P resources (such as rock phosphate) for fertiliser production and the environmental impacts of inefficient use (Scavia *et al.*, 2014). Therefore, the application of alternative P sources is required to support sustainable agricultural production; one such alternative is the use of P-rich organic materials (Cavagnaro, 2015).

Agriculturally generated organic wastes such as animal manures can be rich in P, accounting for 0.3 – 4.0 % of the material dry weights (Pagliari and Laboski, 2012). However, the P in these materials is present in diverse forms, including plant-available orthophosphate and organic P substances that differ in their P release in soil (Mackay *et al.*, 2017b; Noack *et al.*, 2012). As the P mineralisation processes that govern the transformation of organic P forms to plant-available (mineral) P forms are controlled by numerous soil factors (Bünemann, 2015), the amount of available P supplied to plants is difficult to predict and may not be sufficient and/or timed to matched periods of peak plant demand (Dey *et al.*, 2019). Therefore, if organic materials are to be used as P fertilisers, a comprehensive understanding regarding their behaviour in the soil-plant continuum is needed.

One option for the efficient and more predictable use of organic waste materials as a source of P in agriculture, is to blend them with mineral P sources (Gale *et al.*, 2014). That is, in addition to reducing the use of P chemical fertilisers, the available P from the chemical fertiliser and partly from the organic material can be quickly delivered to sustain initial growth (Grant *et al.*, 2001), and the P required for subsequent plant growth will be replenished as the organic material is mineralised (Malik *et al.*, 2013). While this approach has shown some promise (Wei *et al.*, 2016), the rate of inorganic P substitution may affect crop yields and P use efficiency (Xin *et al.*, 2017). Thus, further studies are needed to obtain more understanding on the impacts of alternative organic P sources.

Supplying P to plants in an available form when and where it is needed is only part of plant P acquisition - the plants also need to assimilate the P. Arbuscular mycorrhizal (AM) fungi have been targeted in various studies for the improvement of plant P acquisition (see Chiu and Paszkowski (2019) for review). Arbuscular mycorrhizal fungi (AMF) are ubiquitous in soils, and form a symbiosis within roots of the vast majority of terrestrial plant species (Smith and Read, 2008; Willis *et al.*, 2012). The P acquisition of the host plants can be improved by various mycorrhizal modes of action. For example, mycorrhizal fungal hyphae can extensively expand the scavenging areas of available P in soils, and can thus increase P transported into the plant (Mai *et al.*, 2019). In addition, AMF can directly release

phosphatases to hydrolyse organic P compounds in soils (Feng *et al.*, 2002; Koide and Kabir, 2000) or dissolve relative insoluble P (Joner *et al.*, 2000; Pel *et al.*, 2018). Furthermore, mycorrhizal fungi can indirectly contribute to increase plant P acquisition by inducing growth of phosphate solubilizing bacteria (Jiang *et al.*, 2020; Zhang *et al.*, 2018) or enhance phosphatase activity of roots (Amaya-Carpio *et al.*, 2009). However, plants exchange C in return for P received from AMF (Pearson and Jakobsen, 1993). Thus, the C-P trade-off between the plant and fungi can result in plant growth responses ranging from positive through neutral to negative (Walder and van der Heijden, 2015; Watts-Williams *et al.*, 2019).

Phosphorus is an important factor controlling the growth of AMF (Gu *et al.*, 2011; Nouri *et al.*, 2014). For example, AM fungal colonisation is generally inhibited by high concentrations of soil P (Watts-Williams *et al.*, 2013). In addition, the form of P in soils may also affect the growth of AMF; such as, soils with higher proportion of sparingly soluble P and/or phytate can enable higher mycorrhizal fungal colonisation compared to soil with high soluble orthophosphate (Pel *et al.*, 2018). Therefore, applying P originated from organic materials may benefit mycorrhizal performance and contribute to sustainable agricultural development where not only plant yields, but also soil health are important to land management (Rillig *et al.*, 2019).

Taken together, there has been increasing attention on the development of alternative fertilisers that use organic materials in combination with chemical P fertilisers. Given that the form of P supplied to plants can greatly impact the dynamics of plant-available P in the soil over time, it may be possible to optimise P supply to plants by applying the mixtures of multiple P sources to the soil (Frazão *et al.*, 2019). However, there is a lack of understanding of the contribution of AMF to plant P uptake in conjunction with these combined fertilisers. Therefore, here we present results of an experiment in which we supplied plants with the same amount of total P, but in varying ratios of inorganic and organic P sources. Specifically, we aimed to answer two research questions:

1. To what extent are the growth and nutrition of tomato plants affected by soil amended with different ratios of inorganic P and organic material?
2. Do changes in the ratio of inorganic to organic P sources affect the mycorrhizal formation and functioning of tomato plants?

## 2. Materials and Methods

### *2.1. Soil and phosphorus source preparation*

The soil used in this study was a sandy loam, Urrbrae red-brown earth (Alfisol), collected from the 0-10 cm soil layer in the Waite Arboretum, South Australia (S34°58’01’’, E138°37’46’’). The soil was passed through a 2 mm sieve to remove any coarse debris and to homogenize. After soil was air-dried,

it was mixed thoroughly with a coarse/fine (7:3 w/w) sand mix in a 1:9 ratio (soil/sand mix, w/w) using a cement mixer. In addition to native AMF propagules from the collected soil, the AMF *Rhizophagus irregularis* WFVAM10 (DAOM181602) was added at 5% (w/w) to the soil/sand mix. The *R. irregularis* was grown in pot cultures of 9:1 w/w soil/sand mix using *Tagetes patula* as a trap plant (Walker and Vestberg, 1994). Accordingly, this soil substrate provides a low P content that sustains AM colonisation of roots and permits ready extraction of roots at the time of harvest (Ngo and Cavagnaro, 2018). Prior to use, the dry soil/sand mix was irrigated with reverse osmosis (RO) water to 5% soil weight, and incubated at room temperature for 14 days. The purpose of this procedure was to support re-establishment of the soil microbiota, while avoiding impacts of re-wetting soil (Fierer and Schimel, 2003; Kaiser *et al.*, 2015).

Two sources of P fertiliser were used in this experiment: phosphoric acid (inorganic) and chicken litter (organic). Phosphoric acid was used as inorganic P source as it only supplies P nutrient to a soil with minimal effect on soil pH (Mackay *et al.*, 2017b). The chicken litter (straw bedding) was P-rich, containing 16.5 g P $kg^{-1}$ and 2.3 N:P ratio as in previous work (Mackay *et al.*, 2017b).

The experiment included five P source ratio treatments, prepared by substituting a proportion of total P content in the inorganic P source with organic P source. Specifically, a total of 10 mg P $kg^{-1}$ soil was added from inorganic and/or organic P sources in the following ratios: 4:0, 3:1, 2:2, 1:3 and 0:4. The application rate of 10 mg P $kg^{-1}$ soil was selected based on the results of a preliminary experiment testing the response of plant biomass to seven levels of inorganic P apply where where plant biomass was high and mycorrhizal fungal colonisation was not suppressed (see Supp. Figure S1 and S2). As chicken litter contains N, the application of 10 mg P $kg^{-1}$ soil provided 23 mg N $kg^{-1}$ in the 0:4 ratio treatment. In addition, a treatment without addition of either inorganic P or organic amendments were set up as a control to validate plant response to the P fertiliser treatments.

Inorganic and organic P sources were incorporated into the soil individually in separate bags with five replicate each treatment. From each bag a 50 g soil was taken for quantification of plant-available P, electrical conductivity (EC), pH and available nitrogen (N) (see below). The incorporated soils were then used to fill 1.2 L plastic free draining pots (1.4 kg $pot^{-1}$).

## *2.2. Glasshouse experiment*

Two tomato (*Solanum lycopersicum* L.) genotypes were used in this experiment: a mycorrhiza-defective mutant (with reduced mycorrhizal colonisation; *rmc*, hereafter), and its mycorrhizal wild-type progenitor (76R, hereafter) (Barker *et al.*, 1998). The use of two tomato genotypes was designed to investigate AM formation and functioning as the *rmc* plant allows no or limited AM colonisation; however, its biomass is almost identical to the 76R plant when grown in the absence of AMF

(Cavagnaro *et al.*, 2004; Larkan *et al.*, 2013; Watts-Williams and Cavagnaro, 2015). Importantly, this mutant genotype-based approach also avoids the need to sterilise the soil to establish non-mycorrhizal controls, which is especially important in this context where microbially-mediated mineralisation of P underpins soil P supply.

Tomato seedlings were prepared from germinated seeds and raised in a sand substrate until the first true leaves appeared. Each experimental pot had one seedling transplanted into it. All pots were randomly positioned on benches in a glasshouse at the Waite Campus, The University of Adelaide. Conditions in the glasshouse were 22 °C day and 17 °C night with supplemental lighting (1,000 W metal halide lamps) for a 16/8 hours day/night photoperiod. One week after transplanting, a double layer of nylon mesh was placed over the soil of each pot (around the stem) to reduce evaporative water loss. Pots were watered to weight with reverse osmosis (RO) water three times per week to a gravimetric soil moisture content of 10% (Watts-Williams and Cavagnaro, 2018). A modified Long-Ashton solution (P omitted) was applied weekly; each pot received a total of 100 mL solution by harvest (Cavagnaro *et al.*, 2001). No other sources of N were applied to pots.

### *2.3. Soil analysis, and plant harvesting and analysis*

On 42 DAP, a soil core (100 mm length x 10 mm diameter) was taken from each pot at 20 mm distance to stems of the tomato plants. From each soil core, soil was mixed and a proportion of fresh soil was extracted with a 2 M KCl solution (1:5 soil: extractant ratio) for measuring of ammonium and nitrate (Forster, 1995; Miranda *et al.*, 2001). The remaining soil sample was was air-dried for measuring EC and pH (1:5 soil: water suspension) and plant-available (Colwell) P (1:100 soil: extractant) ( (Murphy and Riley, 1962).

Immediately after soil sampling, plants were destructively harvested. Shoots were cut off from roots at the soil surface, then shoot fresh weights were recorded. Roots were gently washed from soil with RO water, then fresh roots were weighed. Fresh roots were sub-sampled for determination of mycorrhizal fungal colonisation by staining with ink and vinegar (Vierheilig *et al.*, 1998) and counting using the grid-line intersect method at 20X magnification (at least 150 intersects were assessed for each sample) (Giovannetti and Mosse, 1980; Rajapakse and Miller Jr, 1992). All of the shoot samples and remaining root samples were oven-dried at 60 °C for 48 hours, then dry weights were recorded. After that, dry plant samples were ground to a fine powder using a puck mill pulveriser machine. Total N concentration was measured using the Dumas method. Total P concentration was measured by the modified phosphovanado-molybdate complex method (Hanson, 1950) using nitric acid aqueous digestion as described previously (Ngo and Cavagnaro, 2018). The contents of N and P in the shoots and roots were calculated by multiplying the concentration of N and P in shoots and roots by shoot and root dry weights, respectively.

As noted above, the experiment included a treatment where plants were grown in soil without any P amendment. As expected, this control treatments produced very small plant dry weights in both 76R (mycorrhizal) and *rmc* (non-mycorrhizal) plants, account for 1.6% to 16% dry weights compared to other treatments (see Supp. Table S1). Thus, the control treatment confirms P-responsiveness of the soil, and were not considered further.

### *2.4. Statistical analysis*

Mycorrhizal growth response was calculated using the individual biomass data of 76R (mycorrhizal) plants and mean biomass of the *rmc* (non-mycorrhizal) plants from the respective treatment (Eq. 1). Mycorrhizal N and P responses were calculated in the same way, with values of total N and total P content in plants, respectively.

$$\text{Mycorrhizal response } (\%) = \frac{\text{76R plant} - \text{mean } rmc \text{ plant}}{\text{mean } rmc \text{ plant}} \text{x } 100 \qquad (1)$$

Data were analysed by generalised linear model (GLM). For starting soil characteristics, mycorrhizal fungal colonisation, and mycorrhizal response (%), the experiment factor was *P ratio treatment*. To determine whether mycorrhizal response means were significantly different from zero (in either positive or negative direction), 95% confidence intervals (CI) were calculated and a treatment mean was deemed to be different where the 95% CI did not overlap zero. Where ANOVA outcomes were significant, Tukey's honestly significant difference (HSD) test was used to explore the differences among treatments with $\alpha$ level 0.05. All data analyses were performed in the R statistical environment version 3.5.1 (2018-07-02) -- "Feather Spray", using the “readr”, “dplyr” and “agricolae” packages.

## 3. Results

### *3.1. Soil characteristics*

At the start of the experiment, whereas all soil treatments received the same amount of total P, plant-available P concentration in the soil was decreased in the treatment with higher proportion of added organic P source (Table 1). In contrast, the addition of organic P source increased ammonium, and to a lesser extent pH of the soil. However, soil EC and nitrate did not differ between the different P source treatments (Table 1).

Table 1. Characteristics of the soil at the time of planting after incorporating inorganic P and organic material (chicken litter). The P source ratio treatment refers to the proportion of P supplied as phosphoric acid relative to the proportion of P supplied as organic material. Each P treatment received 10 mg total P $kg^{-1}$. Values are mean ± SE, *n* = 10. Within column, Means with at least one similar letters are not significantly different at the P <0.05 level.

| P source ratio | Plant-available P (mg $kg^{-1}$) | pH | EC (µS $cm^{-1}$) | Ammonium (mg $kg^{-1}$) | Nitrate (mg $kg^{-1}$) |
|---|---|---|---|---|---|
| **(4:0)** | 11.79±0.45(a) | 5.41±0.02(b) | 34.70 ±1.91 | 0.56±0.04(c) | 31.29 ±0.45 |
| **(3:1)** | 9.58±0.42(b) | 5.39±0.01(b) | 35.20 ±1.09 | 1.27±0.06(c) | 31.77 ±0.36 |
| **(2:2)** | 7.57±0.70(c) | 5.43±0.01(b) | 39.54 ±2.32 | 2.25±0.32(b) | 31.57 ±0.33 |
| **(1:3)** | 6.83±0.42(c) | 5.60±0.01(a) | 40.20 ±1.32 | 2.77±0.05(ab) | 30.23 ±0.34 |
| **(0:4)** | 5.79±0.48(c) | 5.67±0.03(a) | 40.12 ±2.45 | 3.24±0.23(a) | 31.06 ±1.08 |

At the time of harvest, the concentration of plant-available P in soils was reduced in the 4:0 and 3:1 P ratio treatments, but the reduction was small in other P ratio treatments compared to at the start of the experiment (Table 1, Fig. 1). Specifically, at the time of harvest, the concentration of plant-available soil P was highest in the 3:1 P ratio treatment, lowest in the 0:4 P ratio treatment. Irrespective of P ratio treatment, plant-available P in the soil was lower in pots with 76R (mycorrhizal) plants compared to *rmc* (non-mycorrhizal) plants.

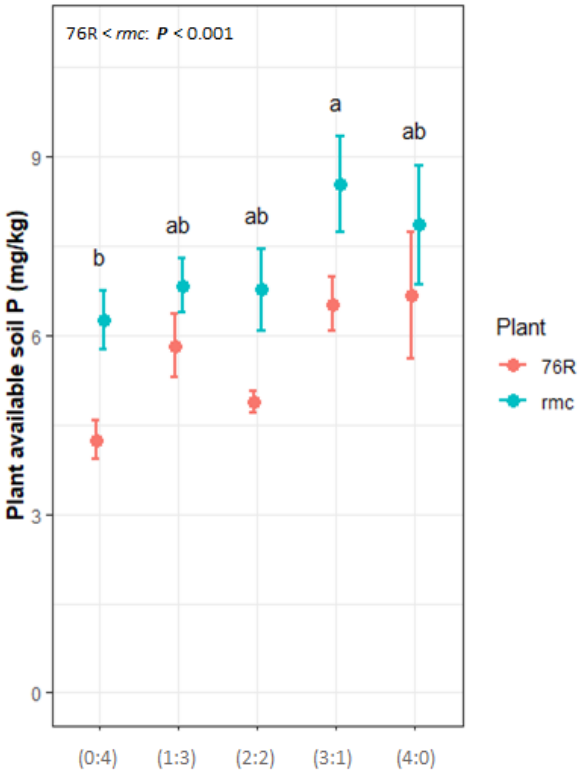


Fig. 1. Plant-available (Colwell-extractable) soil P concentration of pots that hosted 76R (mycorrhizal) plants and *rmc* (non-mycorrhizal) plants on the day of harvest at five P source ratio treatments. Values are mean ± SE, *n* = 5. Letters above bars shows HSD test values of P source ratio treatments. Means with at least one similar letter are not significantly different at the P <0.05 level.

### 3.2. *Arbuscular mycorrhizal fungal colonisation*

Arbuscular mycorrhizal fungal colonisation was not observed in roots of the *rmc* (non-mycorrhizal) plants in any of the P ratio treatments. In the 76R (mycorrhizal) plants, different P ratio treatments did not result in differences in mycorrhizal fungal colonisation (Fig. 2). That is, percent mycorrhizal fungal colonisation was similar in treatments where P was supplied solely as inorganic P or as organic material, and any combination therein, with mean value of 57.7 % root length colonised.

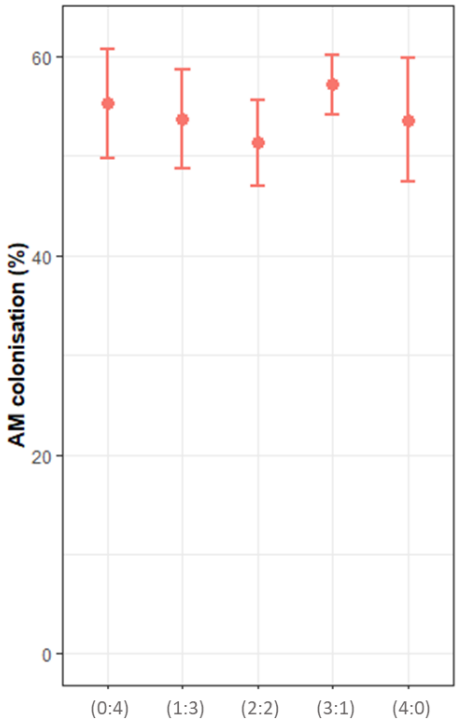


Fig. 2. Percent root length arbuscular mycorrhizal colonisation in 76R (mycorrhizal) plants grown at five different P source ratio treatments. Values are mean ± SE, n = 5.

### 3.3. *Growth and nutritional status of tomato shoots and roots*

Shoot dry weight was significantly higher in the 0:4 (organic P alone source) and 1:3 treatments compared with the 4:0 treatment (inorganic P alone source), with the other treatments being intermediate (Fig. 3a). The pattern of total P content (Figure 3b) and total N content in shoots (Fig. 3c) were similar to the pattern of shoot dry weights, with the highest and lowest P and N nutrition contents in the 0:4 and 4:0 P ratio treatments, respectively. Irrespective of P ratio treatment, the shoot dry weight, shoot P content and shoot N content of the two tomato genotypes were not different.

In contrast to shoots, P ratio treatment did not affect root dry weight, root P content and root N content (Fig. 3 c-d). However, whereas root biomass and root N content were matched between two tomato genotypes, root P content was higher in the 76R (mycorrhizal) plants compared to *rmc* (non-mycorrhizal) plants.

### 3.4. *Mycorrhizal growth and nutrition responses*

Different P ratio treatments did not alter mycorrhizal growth response (MGR) of the tomato 76R plants (sum of shoot and root) (Fig. 4a). Similarly, mycorrhizal N response (MNR) was neutral and was not different in any of P source ratio treatments (Fig. 4c). In contrast, positive mycorrhizal P response (MPR) (Fig. 4b) was observed in all treatments, except for the 1:3 ratio treatment with neutral effect.

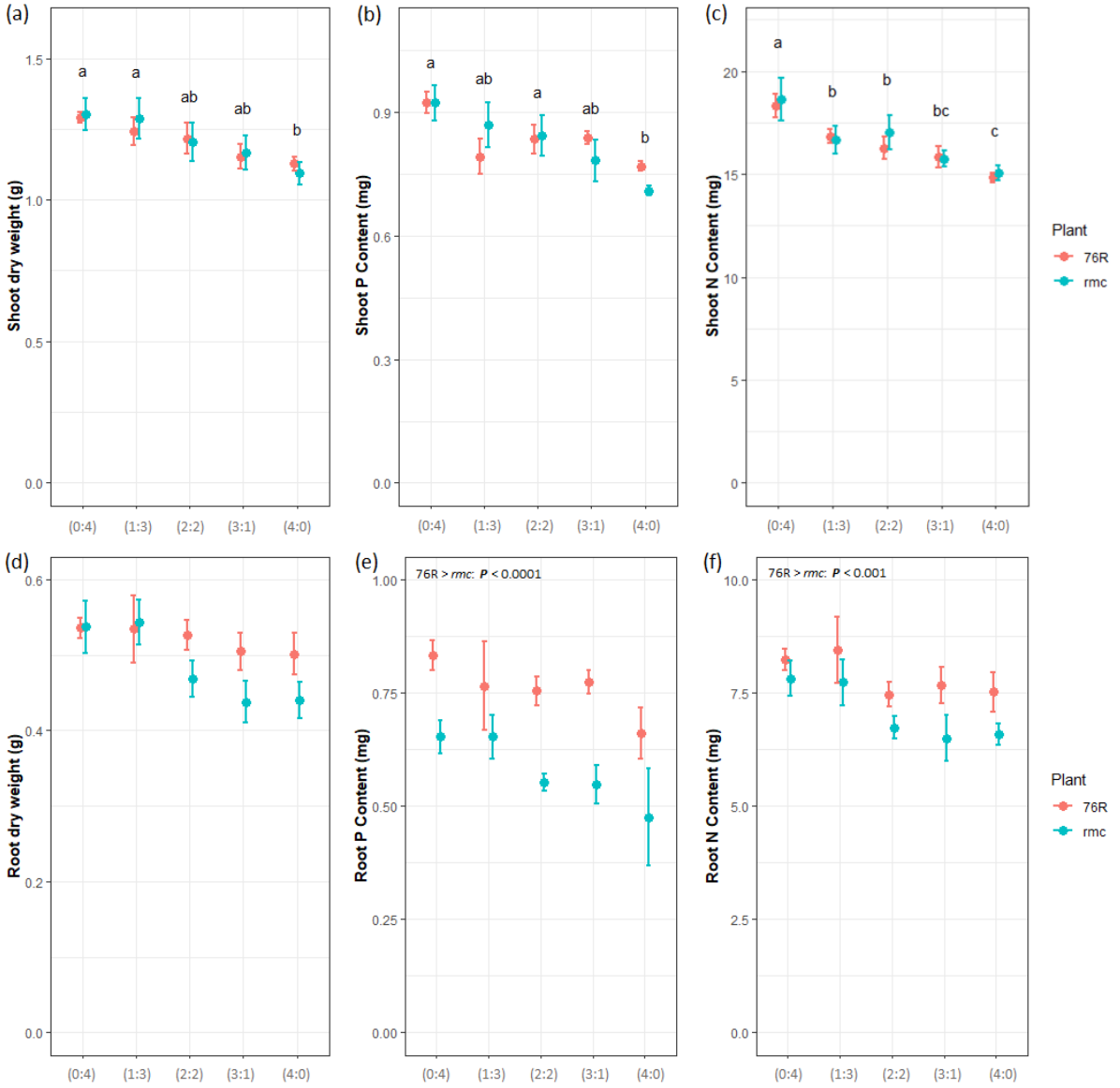


Fig. 3. Dry weight, P content and N content in shoots and roots in 76R (mycorrhizal) plants and *rmc* (non-mycorrhizal) plants grown at five different P source ratio treatments. Values are mean ± SE, *n* = 5. Letters above bars shows HSD test values of P source ratio treatments. Means with at least one similar letter are not significantly different at the P <0.05 level.

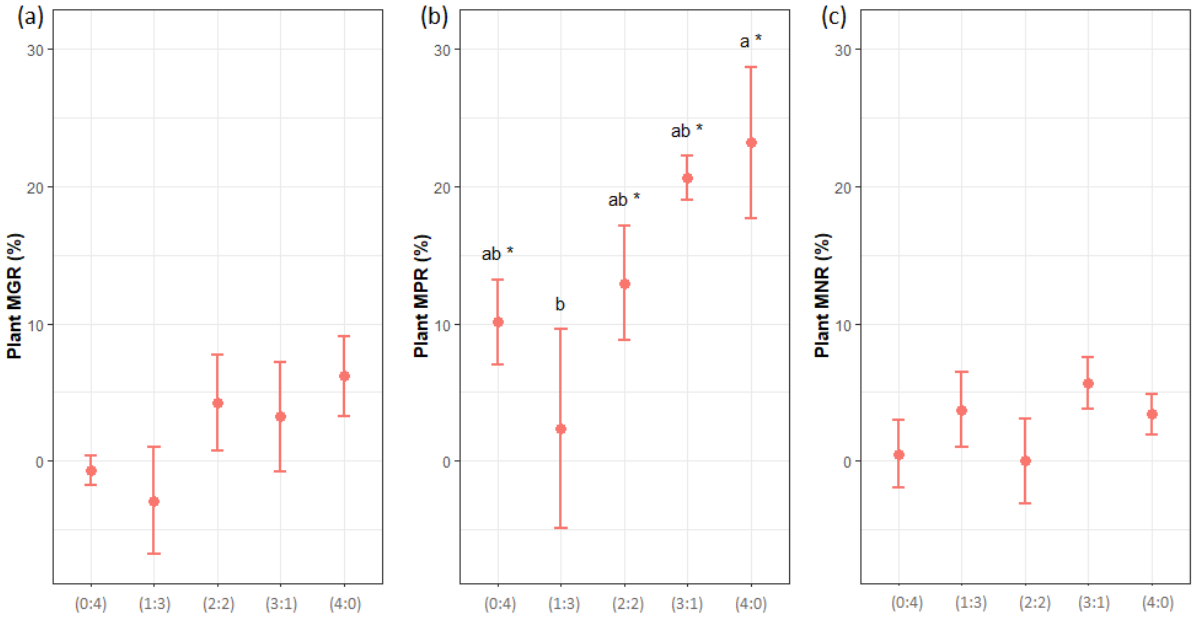


Fig. 4. Mycorrhizal growth response (MGR), Mycorrhizal P response (MPR) and mycorrhizal N response (MNR) in 76R (mycorrhizal) plants grown at five different P source ratio treatments. Values are Mean ± SE, *n* = 5. Means with at least one similar letter are not significantly different at the P <0.05 level. Asterisk indicates means are different from zero at 95% CI.

## 4. Discussion

The source of P (inorganic, organic or mixed) had a clear impact on the growth, nutrition and mycorrhizal responses in tomato plants. The higher P concentration in the soil obtained after applying the inorganic P alone source did not translate to greater plant growth and P uptake. However, the fate of available P release P from the organic alone and combined P sources provided P that better support plant P uptake and hence plant growth. The results highlight the increasing trend toward positive mycorrhizal P response with the use of the inorganic P alone source.

### *4.1. Effect of P sources on soil P availability*

Soil properties measured at the start of the experiment changed after incorporating inorganic and organic P sources, including ammonium, pH and plant-available (Colwell) P. Amendment of the soil with chicken litter increased the concentration of ammonium in the soil. This was expected given that chicken litter contains a high proportion of total N as uric acid and urea (Nahm, 2003). However, total available N (sum of ammonium and nitrate) did not change among different P source ratio treatments as proportion of ammonium was negligible compared to nitrate. The inorganic P alone treatment reduced the pH compared to the organic amendment alone treatment. However, this small difference (up to 0.28 units) was not likely to have had an effect on P adsorption (Scanlan *et al.*, 2016), tomato plant growth (Dysko *et al.*, 2008) or the formation and function of mycorrhizas (Abbott and Robson, 1985; Habte, 1995).

Although the total P added to all treatments was the same, the soil amended with organic material had lower plant-available P content. This can be explained by the fact that the organic material conserves P in both readily available forms and slow release forms where they need to be mineralised before becoming available to plants (Mackay *et al.*, 2017b). At the time of harvest, the concentration of P in soil was high in the inorganic P alone treatment, indicating that a large proportion of available P remained in the soil which may be due to low plant P uptake and high P sorption and precipitation effects (Asomaning, 2020; Hedley and McLaughlin, 2005; Holford, 1997). In the other treatments involving organic material, the concentration of plant-available soil P was also reduced. However, the lower reduction compared to the inorganic P alone treatment could be explained by the replenishment of P from mineralisation of the organic P source (Malik *et al.*, 2013). Thus, different P sources could affect plant growth and nutrition by their different reactions in the soil.

### *4.2. Growth and nutritional responses of tomato plants to different P sources*

Plant-available P in the soil was within an acceptable range for the growth of 76R and *rmc* tomato plants; for example, as previously reported in an organic farming practice (Bowles *et al.*, 2016). However, here we found no linear correlation between plant growth (and plant P content) and plant-available soil P at the start of the experiment, as similar to previous research in low-P soils (Kulhánek *et al.*, 2007; Mackay *et al.*, 2017b). The growth of plants increased as a higher proportion of organic material was added, which may be due to the fate of P released from organic alone and combined P source, supporting plant P uptake. For example, the greater P acquisition by plants grown in the organic alone and combined P sources than in the inorganic P alone source may be explained by sufficient initial supply of plant available P (as orthophosphate, Mackay *et al.* (2017b)), followed by mineralisation of organic P over the growth cycle of the plants (Malik *et al.*, 2013). In contrast, much of the P supplied in the inorganic treatment was likely rendered unavailable to plants, due to absorption and precipitation reactions (Barrow and Debnath, 2014) or microbial immobilisation (Marschner *et al.*, 2011). Taken together, this research provides a strong case for using organic materials, or hybrid sources of fertilisers, to supply P to plants.

### *4.3. Arbuscular mycorrhizal colonisation and responses in different P sources*

While different P source ratios changed plant-available P concentration of the soil, they did not affect AM colonisation of roots, which did not follow the commonly-reported trend towards adverse effect of increased soil P availability on AM colonisation (Gu *et al.*, 2011). However, the same effect was reported in maize where there was no influence of isolated P sources compared to combined inorganic P and organic materials on mycorrhizal root length colonisation (Carrenho *et al.*, 2007). The high rate of AM colonisation in all treatments could be due to the low level of plant-available soil P in all

treatments. For example, while plant-available P of the soil in this experiment was lower than a threshold level (up to 20 mg P $kg^{-1}$ soil) for maximum mycorrhizal fungal colonisation (Shukla *et al.*, 2012), AM colonisation in poor P soil can be induced with the increase of soil P concentration (Bolan *et al.*, 1984). In addition, the promotion of mycorrhizal fungal colonisation in the organic P alone and combined treatments could be due to the presence of substances such as phytate and lecithin, which have been shown to be associated with higher levels of mycorrhizal fungal colonisation in red clover (Feng *et al.*, 2003), wheat (Mackay *et al.*, 2017b), and chickpea (Alloush *et al.*, 2000). Therefore, in a low P input application, the formation of mycorrhizas could possibly be unaffected by different P sources, which is in contrast to what is expected with a high P input application (Mackay *et al.*, 2017a).

Arbuscular mycorrhizal associations increased plant P uptake, especially in the inorganic P alone treatment, despite the higher plant-available P that was measured in the soil. This indicated the benefit of AMF in supporting plants to overcome the limitation of roots in scavenging for available P, where P may be less accessible to roots due to P sorption and precipitation, as be seen in the inorganic P alone treatment. In contrast, the tomato plants were less dependent on AMF for P acquisition in the organic alone and combined P source treatments, which could be explained by uptake of P by roots as P was mineralised from the organic P source. While further investigation is required on the dependence of plant on AMF over the plant's life before generalisation can be made, the results suggest that the reliance of the tomato plant on AMF for P acquisition changed depending on plant-available P in the soil, and root development. Although mycorrhizal growth responses were neutral in all P source ratio treatments, they followed the pattern of mycorrhizal P responses, suggesting that the increase in growth of mycorrhizal plants was a consequence of the increase in mycorrhiza-mediated P uptake. However, it is important to note that AM colonisation is not necessarily a good indicator of mycorrhizal function, nor the contribution of the mycorrhizal pathway to P uptake (compared to the direct root pathway) and plant growth (Watts-Williams *et al.*, 2015). Thus, use of isotopic labelling may be required to gain more insights into the C-P dynamics with the different P sources and AM.

## Conclusions

This study demonstrated that plant growth and nutrition were the same, if not better, where a greater proportion of the P was supplied as organic material, than where it was supplied as inorganic P alone. Whereas the formation of AM did not affect plant growth and N nutrition, it did improve plant P nutrition and was more pronounced where P was supplied in the inorganic P alone source. Together, these results suggest that organic materials and arbuscular mycorrhizal associations have the potential to improve P supply to plants in some situations.

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## Supplementary data

Table S1. Characteristics of the soil before incorporating inorganic P source and organic material (chicken litter); and tomato dry weights at the time of harvest in the treatment without addition of either inorganic P or organic amendments. Values are mean ± SE, *n* = 5.

| Plant-available P (mg kg$^{-1}$) | pH | EC (µS/cm) | Ammonium (mg kg$^{-1}$) | Nitrate (mg kg$^{-1}$) | Tomato 76R (g) | Tomato *rmc* (g) |
|---|---|---|---|---|---|---|
| **3.47 ±0.51** | 5.69 ±0.04 | 31.02 ±2.29 | 0.52 ±0.08 | 30.48 ±0.11 | 0.265 ±0.03 | 0.032 ±0.002 |

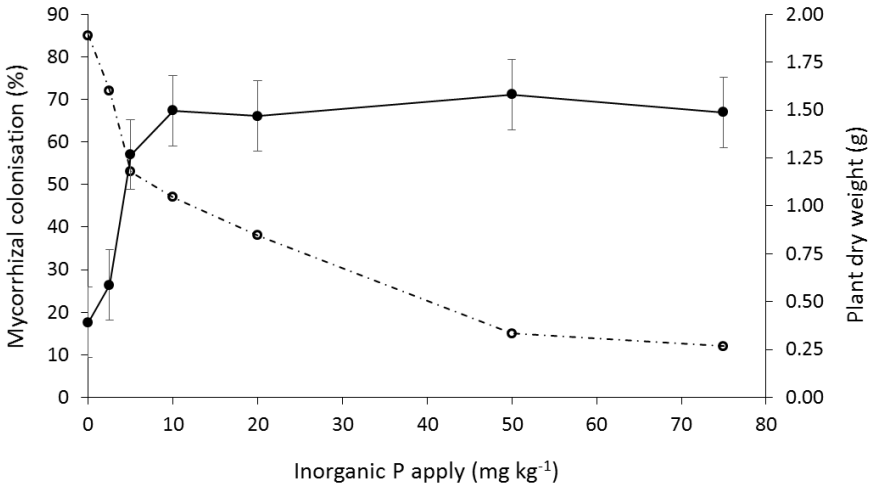


Fig. S1. Plant dry weight of tomato 76R mycorrhizal plant and non-mycorrhizal plants (values were mean, n=2) and mycorrhizal colonisation of tomato 76R mycorrhizal plants (n=1) in different inorganic P apply.

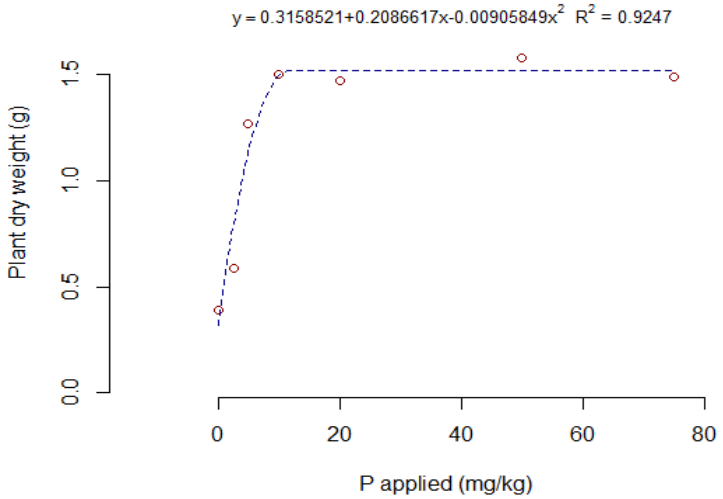


Fig. S2. Quadratic plateau model for describing plant dry weight responded to P applied (values are means, n=2). Parameters and the coefficient of determination ($R^2$) of the model are estimated using quadratic linear model in R. Quadratic response model was defined by y ~ (a + b * X + c * I($x^2$)) * (x <= -0.5 * b/c) + (a + I(-$b^2$/(4 * c))) * (x > -0.5 * b/c). Where y is plant dry weight (g), x is the rate of P apply (mg kg$^{-1}$), a, b and c are parameters of the model. Predicted critical P apply for optimum rate of plant dry weight was defined by the value of x = -0.5 * b/c = 11.47).

# CHAPTER 3: MYCORRHIZAL GROWTH AND PHOSPHORUS RESPONSES OF TOMATO DIFFER WITH SOURCE BUT NOT APPLICATION RATE OF PHOSPHORUS FERTILISERS

# Statement of Authorship

| | |
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## Principal Author

| | | | |
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| Name of Principal Author (Candidate) | Thi Thanh Hue Ngo | | |
| Contribution to the Paper | Contributed to the development of ideas, performed glasshouse and laboratory work, interpreted data, wrote the manuscript and acted as corresponding author. | | |
| Overall percentage (%) | 90% | | |
| 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. | | |
| Signature | | Date | 10/06/2021 |

## 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.

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# Mycorrhizal growth and phosphorus responses of tomato differ with source but not application rate of phosphorus fertilisers

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ABSTRACT

Phosphorus (P) can be added to the soil from various sources (e.g., chemical fertilisers and organic materials), and P fertilisation affects mycorrhizal colonisation and function in plants. While arbuscular mycorrhizal symbiosis is an integral part of most crop plants, there is a gap in understanding mycorrhizal growth and nutrition responses in relation to different sources of P at similar and variable application rates. Here we explore the impacts of different P sources (solely inorganic, mixed, or solely P-rich organic material), applied at three P application rates, on plant growth, nutrition and mycorrhizal responses. Tomato plants (arbuscular mycorrhizal and non-mycorrhizal plants) were grown in a soil amended with 10, 20 and 40 mg P $kg^{-1}$. We found that the solely inorganic P source affected mycorrhizal and non-mycorrhizal plants differently to the solely P-rich organic source, as did the combination of the two, even with P application rates matched between different sources. The solely inorganic P source consistently favoured mycorrhizal plants, whereas mycorrhizal plants performed less successfully than non-mycorrhizal plants in the solely P-rich organic source. However, mycorrhizal and non-mycorrhizal plants responded equally in the soil where the mixed P source was added. The results indicated that blending inorganic and organic P sources could be used to mitigate negative effects of AMF on plant growth and P nutrition compared to using solely P-rich organic material.

## 1. Introduction

A shift towards ecological intensification is gaining increased attention globally (Pretty et al., 2018). This includes encouraging the use of organic fertilisers (Zhang et al., 2017) and improving reliance on biologically-regulated nutrient supply systems (Cavagnaro, 2015; Rillig et al., 2016). Organic materials such as chicken litter are sustainable substitutions for chemical fertilisers (Reganold and Wachter, 2016); however, one argument against their use as fertilisers is that they may yield less crop products than inorganic fertilisers (Cooper et al., 2018). While inorganic fertilisers can boost crop yields, long term use can result in soil degradation; for example, reduction of soil organic matter (Bhatt et al., 2019), suppression of soil microbial diversity (Cui et al., 2018), and facilitate nutrient losses to the ground water and atmosphere (Roberts and Johnston, 2015). On the other hand, the combined use of inorganic fertilisers and organic materials may reduce the amount of inorganic fertiliser that needs to be added to soils (Mackay et al., 2017a), improve plant yields and soil fertility (Liu et al., 2017; Qaswar et al., 2020) while controling potential risks of nutrients loss due to leaching (Vanden Nest et al., 2014). Thus, the combined use of inorganic fertilisers and organic materials may be one option for the ecological intensification of agriculture.

Phosphorus (P) frequently limits plant growth in many farming systems, as it often presents in low concentration, has low mobility, and is highly reactive with soil elements (Shen et al., 2011). As P sources are finite and non-renewable, P-rich organic materials, such as chicken litter, have been suggested as important alternative sources of P for agricultural use (Calabi-Floody et al., 2018). In addition, organic P sources not only contain P, but also other essential elements, including but not limited to nitrogen and carbon (Sager, 2007). Thus, an application of the same amount of total P in the form of an organic amendment, compared to a purely inorganic P source, will bring with it other benefits. However, P in organic materials is presented in different chemical forms (e.g., phytate, nucleic acids), that are gradually

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mineralised before being taken up by plants (Cooper et al., 2018). While there are many factors affecting the fates of P turnover in soils (Stutter et al., 2015), P inputs in agriculture need to be carefully managed. Phosphorus fertilisers have been found in some instances to be applied in higher quantities than the recommended rates (Valkama et al., 2009). However, high application rates of P fertilisers can lead to P losses to the environment (Khan et al., 2018; Tran et al., 2020) and low economic returns (Lun et al., 2018). In addition, the source and quantity of P inputs can affect soil microbial interactions, P mineralisation and uptake by plants (Clausing and Polle, 2020; Gumiere et al., 2019). Therefore, to ensure sustainable use of P and crop production, it will be important to understand how to most efficiently use different P sources (e.g., organic vs inorganic). Not only does this require knowledge of the behaviour of different P sources in the soil (Mackay et al., 2017b), but also the ways in which plants assimilate P.

Arbuscular mycorrhizal fungi (AMF) are a group of fungi that are reported to form symbiosis with the roots of the majority (72%) of terrestrial plants (Brundrett and Tedersoo, 2018). In majority of agricultural systems, AMF colonise crop plants and provide important ecological services such as nutrient cycling, soil fertility and soil biodiversity (Bender et al., 2015; Bowles et al., 2017; Chen et al., 2018). Specifically, AMF enhance the plant uptake of water and soil nutrients, especially elements with limited mobility such as P (Li et al., 2013; Xie et al., 2013) and Zn (Coccina et al., 2019). The benefits of AMF to nutrient acquisition are based on the extensive growth of fungal hyphae throughout the soil volume (Mai et al., 2019) and high-affinity nutrient transporters in AMF (López-Pedrosa et al., 2006; Xie et al., 2016). However, in return for mycorrhizal benefits to plants, AMF use carbon compounds, such as sugars and lipids from plant hosts (Jiang et al., 2017; Wipf et al., 2019). Thus, the relationship between AMF and plants is a trade off balance and highly dependent on contexts (Rillig et al., 2019), such as soil management (Singh and Singh, 2019) and soil nutrient availability (Püschel et al., 2016; Raya-Hernández et al., 2020).

The formation and functioning of mycorrhizas is strongly dependent on soil P conditions (Schmitz and Harrison, 2014). For example, high levels of available P in soil can adversely affect early symbiotic signalling events (via the plant hormone strigolactone), subsequently resulting in less successful mycorrhizal colonisation and down-regulated AM-related gene expression (Tsuzuki et al., 2016). As a consequence of these changes in signalling, nutrient uptake via the mycorrhizal pathway can be reduced (Clausing and Polle, 2020). However, the critical values of available soil P for mycorrhizal colonisation inhibition are generally higher than those for optimum plant growth (Bai et al., 2013; Deng et al., 2017). Thus, suitable P fertilisation in combination with mycorrhizal colonisation could improve plant yield and P use efficiency. In addition, it is not only the amount of P applied that can affect mycorrhizal colonisation and functioning, but also the forms of P supplied. For example, sources of P affect level of root colonisation by the AMF (Mackay et al., 2017a), and mycorrhizal community composition between conventional and organic managements (Aldrich-Wolfe et al., 2020; Dai et al., 2014). While AMF may provide a sustainable solution to improve plant growth and plant P uptake, it is important to understand effects of AMF on plant growth and P nutrition when using different sources of P fertilisers at various application rates. This understanding is necessary to develop effective P fertiliser strategies that are compatible with both mycorrhizal fungi and plants, and hence, contribute to sustainable agricultural production. While tomato plants typically show moderate levels of arbuscular mycorrhizal (AM) colonisation (Cavagnaro and Martin, 2011), they are very sensitive to the presence or absence of AM associations under certain environmental conditions, such as low light intensity (Marschner and Timonen, 2005), soil water stress (Sun et al., 2018; Xu et al., 2018) and soil nutrient conditions (Bowles et al., 2016; Saia et al., 2020; Watts-Williams et al., 2019). Therefore, we sought to explore the impacts of different P sources and application rates on soil P availability, tomato plant growth and nutrition, and the formation and functioning of arbuscular mycorrhizas. Specifically, our hypotheses were that:

i. Inorganic P sources will provide higher plant-available P to a soil compared to mixed and organic P sources, and the difference among P sources will be proportionate with the increase in P doses;
ii. Plant P content and plant biomass will match the pattern of available P content of a soil;
iii. The presence or absence of AM associations will alter plant growth and P uptake in response to both P source and P dose.

## 2. Materials and methods

### 2.1. Soil material

A sandy loam soil was collected from the 0–10 cm layer from the Waite Arboretum, South Australia (S34°58′01″, E138°37′46″) in 2017. The soil was air-dried and passed through a 2 mm sieve. The sieved soil was then mixed thoroughly with fine sand in a ratio of 1:9 (soil: fine sand; *w/w*) to provide a low P concentration (see below) that sustains AM fungal colonisation of roots and permits ready extraction of roots at the time of harvest (following Cavagnaro et al. (2007)). In addition, dry AM inoculum was added to the soil sand mix at 5% (*w*/w), which contained spores and hyphae of *Rhizophagus irregularis* WFVAM10 (synonymous with DAOM181602) and colonised root fragments. The WFVAM10 isolate has previously shown to colonise the roots of the 76R tomato genotype, but not those of the *rmc* genotype (Gao et al., 2001; Manjarrez et al., 2008). Specifically, 1330 g of the dry soil/sand mix was packed in each undrained plastic cylinder pot with the incorporation of 70 g dry AMF inoculum. Prior to use in the experiment, pots were stabilised for 21 days at room temperature with 5% water content to re-establish the soil microbiota and to minimise potential impacts of re-wetting soil before transplantation (Kaiser et al., 2015). Characteristics of the stabilised soil were 3.2 ± 0.42 mg $kg^{-1}$ plant available (Colwell) P, 3.19 ± 0.19 mg $kg^{-1}$ ammonium ($NH_4^+$), 15.57 ± 1.46 mg $kg^{-1}$ nitrate ($NO_3^-$); the soil also had pH and electrical conductivity (EC) values of 5.69 ± 0.04 and 31.02 ± 2.29 μS $cm^{-1}$, respectively.

### 2.2. Experimental treatments and design

The experiment was set up in a glasshouse at the University of Adelaide Waite Campus from September to October 2018 (Austral Spring). The experiment included three P sources: solely inorganic P source (diluted phosphoric acid), solely organic P source (chicken litter), or the (1:1) mix of two materials based on the contribution of total P from the inorganic and organic P sources. In addition, each P source was tested at four total P doses: 0, 10, 20 and 40 mg P $kg^{-1}$ dry soil. Specifically, diluted phosphoric acid was used as it introduced solely P mineral to the soil and the diluted solution previously shown to have no significant impact on soil pH (Mackay et al., 2017b). The diluted phosphoric acid was prepared by adding 10.51 mL of 85% phosphoric acid to reverse osmosis (RO) water to make up total 500 mL volume. A 5 mL aliquot of this diluted solution provided 56 mg P per pot (equal to 40 mg P $kg^{-1}$ soil). The diluted phosphoric acid solution was further diluted with RO water to achieve three concentrations: 5, 10 and 20 mg P $kg^{-1}$ soil in a 5 mL volume. The chicken litter (straw as bedding material) was dried at 40 °C and unground prior to use; the chicken litter had 9.9C/N ratio, 38.7 g N $kg^{-1}$ and 16.5 g P $kg^{-1}$ as presented in Mackay et al. (2017b). In general, chicken litter contains P and a suite of other nutrients (e.g., N, S and Zn (Redden and Wallis, 2015)). When adding chicken litter on the basis of total P, there is the option of attempting to balance other nutrients (through the addition of inorganic salts), or to view the additional nutrients that are co-applied with the P in the chicken litter as a further potential benefit of supplying P in this manner. In this study, we took the latter approach (see discussion), consistent with the earlier study of this nature (Mackay et al., 2017b). Thus, phosphoric acid (5 mL), chicken litter and a further 65 mL of RO water were mixed thoroughly into the pots, indicating 0 DAP of the

experiment. Supplementary Table S1 shows mean values for soil characteristics of different treatments at 0 DAP. The following day, 50 g soil was sampled from each pot for the quantification of $NH_4^+$, $NO_3^-$, plant-available (Colwell) P, EC and pH before growing tomato plants.

Tomato (*Solanum lycopersicum* L.) genotypes 76R (able to be colonised by AMF) were planted in pots. In addition, a matched set of soil pots was used to grow tomato 76R genotype corresponding to mycorrhizal-defective mutant, *rmc* genotype (unable to form AM in roots with *R. irregularis* WFVAM 10, referred to hereafter as non-mycorrhizal plants). The use of two tomato genotypes enabled to study the effects of AMF on plant growth and nutrition while maintaining natural conditions of soil (Watts-Williams and Cavagnaro, 2015). Prior to planting, tomato seeds were surface sterilised by 4% NaOCl for 15 min and pre-germinated in clean sand substrate for three weeks. On the first DAP of the experiment, one 3-week-old tomato plant was transplanted into each pot after soil samples were taken. Each treatment was set up with five biological replicates, giving a total of 120 pots (3 P ratios × 4 P doses × 2 tomato genotypes × 5 replicates). All pots were randomly positioned on benches in the glasshouse and re-randomised weekly. The temperature in the glasshouse was 22.4 °C day and 19.9 °C night with supplemental lighting (1000 W metal halide lamps) for a 16/8 h day/night photoperiod. From 7 DAP to 35 DAP, all experimental pots were supplied weekly with 20 mL modified Long Ashton solution (N added and P omitted) (Cavagnaro et al., 2001; Hewitt, 1952). In addition, pots were watered daily by RO water to maintain a (gravimetric) water content at 10% throughout experiment. As a result, each plant received a total of 100 mL modified Long Ashton solution (P omitted) and 50 mg $NH_4NO_3$ as ammonium nitrate solution by harvest.

### 2.3. Plant and soil analysis

On 42 DAP, a soil core (50 g) was taken from each pot for physical and chemical analysis. Subsamples of fresh moist soil were extracted by 2 M KCl solution (1:5 soil: extractant ratio) for measuring $NH_4^+$-N concentration (Forster, 1995) and $NO_3^-$-N concentrations (Miranda et al., 2001). Electrical conductivity (EC) and pH were measured on air-dry soil samples in a 1:5 soil:water suspension. Plant-available (Colwell) P was measured in an aliquot of a 0.5 M $NaHCO_3$ soil extraction (1:100 soil:extractant) (Murphy and Riley, 1962).

After taking soil samples, plants were destructively harvested on 42 DAP; shoots were cut off from roots at the soil surface, then shoot fresh mass was recorded. Roots were gently washed free of attached soil with RO water, then fresh roots were weighed. Approximately 200 mg of fresh roots were sub-sampled and fixed overnight in 50% ethanol. The fixed roots were rinsed and then cleared by being submerged in 10% KOH solution at room temperature for five days. Once sufficiently cleared, the roots were rinsed thoroughly with RO water and then stained by heated 5% ink-vinegar solution at 60 °C for 10 min (Vierheilig et al., 1998). Following staining, roots were de-stained in acidified water overnight and then stored in 50% glycerol solution. Then the percentage of mycorrhizal root length colonisation was then estimated using the gridline intersect method under a stereo microscope at 100 × magnification (Giovannetti and Mosse, 1980).

The shoot and remaining root materials were oven-dried at 60 °C until constant weights were obtained, then they were weighed to obtained dry mass measurements. The dry shoot and root samples were ground to a fine powder by a ring and puck mill pulveriser. Total shoot N concentration was measured using the Dumas method by APAL service (http://www.apal.com.au/ last accessed March 2021). Total shoot and root P concentration was measured by the ICP-OES service (https://www.adelaide.edu.au/fertiliser/ last accessed March 2021) using nitric acid aqueous digestion as described previously (Wheal et al., 2011). The contents of N and P in the shoots (and roots) were calculated by multiplying the concentration of N and P in shoots (or roots) by shoot (or root) dry weights.

### 2.4. Calculations and statistical analysis

Tomato plants demonstrated strong growth responses to P addition. Specifically, without P applied to soil, the average dry biomass of mycorrhizal and non-mycorrhizal plants were 0.39 and 0.26 g, respectively. Whereas with P applied to soil, their dry biomass were 4.88 and 4.89 g, respectively. For that reason, we present only the P dose treatments at 10, 20 and 40 mg P $kg^{-1}$ hereafter.

Mycorrhizal growth response (MGR) was calculated using the individual biomass data of mycorrhizal plants (76R genotype) and mean biomass of non-mycorrhizal plants (*rmc* genotype) from the respective treatment (Eq. (1)). Mycorrhizal P response (MPR) was calculated in the same way, with values of total P content in shoots. Where the MGR and MPR in shoots significantly differed among treatments, they were further examined for significant differences from zero (in either positive or negative direction) by calculating 95% confidence intervals (CI) of means. A treatment mean was deemed to be neutral if the 95% CI overlapped zero.

$$\text{Mycorrhizal response } (\%) = \frac{\text{76R plant} - \text{mean } rmc \text{ plant}}{\text{mean } rmc \text{ plant}} \times 100 \quad (1)$$

There were three factors used for the plant and soil statistical analyses: P source, P dose, and tomato genotype. The three-way and two-way ANOVA were used to assess the effects of individual factors as well as their interactions, for all measured parameters. Where ANOVA outcomes were significant, Tukey's honestly significant difference (HSD) post hoc test was used to identify differences between means among treatments with $\alpha$ level 0.05. Principal component analysis (PCA) was performed with the plant-available (Colwell) P, shoot dry weight, shoot P content and shoot N content data, in order to reveal any differences in responses of 76R (mycorrhizal) plants and *rmc* (non-mycorrhizal) plants to different P sources and doses. Data analyses were performed in R 3.6.3 (R Core Team, 2020) using the "readr" (Wickham et al., 2020), "dplyr" (Wickham et al., 2018), "agricolae" (Mendiburu, 2020), "ggplot2" (Wickham, 2016), "FactoMineR" (Husson et al., 2020) and "factoextra" (Kassambara and Mundt, 2020) packages.

## 3. Results

### 3.1. Principal component analysis

Principal component 1 (PC1) explained 59.9% of the variation and was driven by shoot dry weight, shoot P content and shoot N content of the tomato plants. Principal component 2 (PC2) explained a further 35.4% of the variation, and plant available soil P and shoot N content were strong positive and negative drivers, respectively. Whereas P dose was mainly separated by PC1, P source was separated by PC2, regardless of mycorrhizal colonisation of roots. In contrast, there was no separation of mycorrhizal and non-mycorrhizal plants as a whole P source and dose treatments. However, within the same P source (inorganic, mixed or organic), principal component analysis showed a clear separation of mycorrhizal and non-mycorrhizal plants along PC1 with a reverse trend between inorganic and organic P sources, and consistent seperation among P dose treatments (Fig. 1).

### 3.2. Plant-available soil P

At the time of planting, plant-available P of the soils was positively correlated with the amount of P added to the soil (Fig. 2, $R^2 = 0.8$). However, whereas the inorganic P source provided 82% of total P in available form, for mixed P source and organic P source provided, 56% and 24% (respectively) of the P applied was present in the available form.

Plant-available P in soils at the time of harvest was lower than that of the beginning of the experiment (Figs. 2 and 3a). However, the reduction was smaller in treatments with high P dose and with organic P

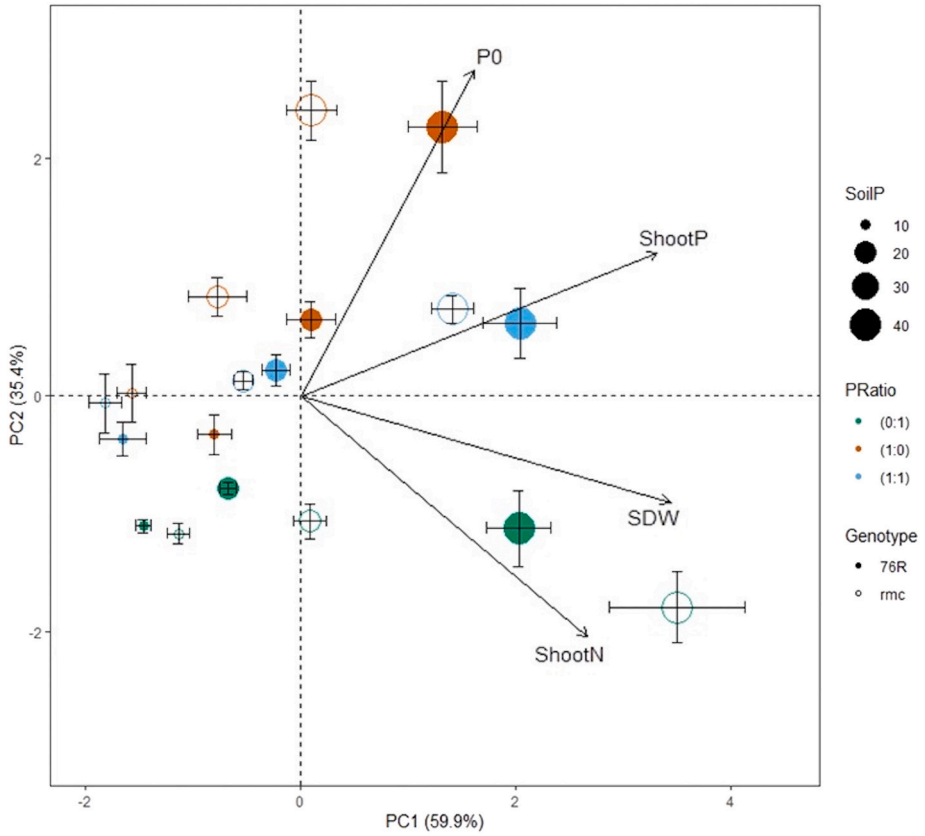


**Fig. 1.** Principal component analysis of tomato functional traits of 76R (mycorrhizal) and *rmc* (non-mycorrhizal) plants grown with P source ratios of (1:0), (1:1) and (0:1) and at soil P doses of 10, 20 and 40 mg $kg^{-1}$. Displayed traits include plant-available (Colwell P) at the time of planting (P0), shoot P content (ShootP), shoot dry weight (SDW) and shoot N content (ShootN). Symbols represent group means and whiskers represent mean standard errors.

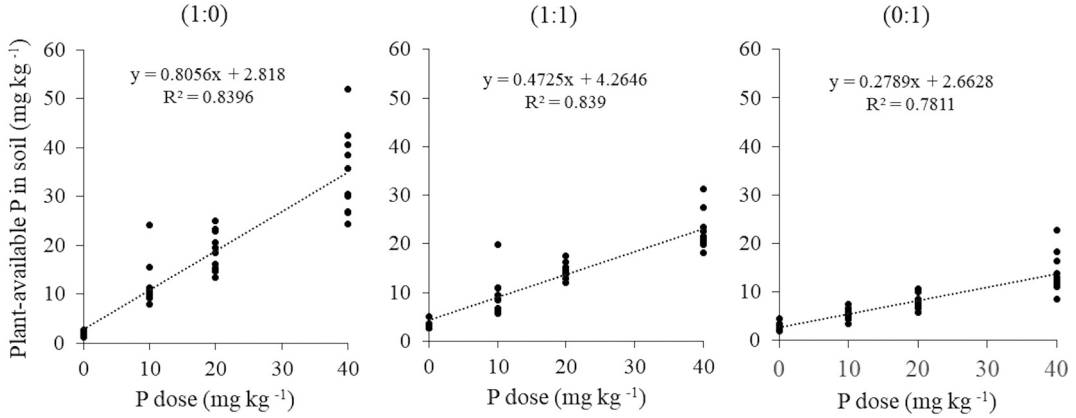


**Fig. 2.** Correlation between P dose and plant-available P in the soils after incorporating solely inorganic P source (1:0), mixed P source (1:1) and solely organic P source (0:1). Dots represent soil samples before planting ($n = 10$).

source. Irrespective of the effects of P source and P dose, there was no effect of mycorrhizal colonisation on plant-available soil P at the time of harvest (Table 1).

### 3.3. Plant growth and nutrition

Phosphorus source and dose had interacting effects on shoot and root dry weights (Fig. 4a, Table 1). Generally, the greater P dose, the higher shoot and root growth; and the effect of P dose was more pronounced in the mixed and organic P sources compared to the inorganic P source.

Phosphorus content of both shoots and roots responded positively to increasing P dose. However, different from the effect on biomass, P contents of shoots were relatively similar within the same P dose, whereas P contents of roots were decreased in response to P source in the order: inorganic P source > mixed P source > organic P source (Fig. 4b, Table 1).

Nitrogen (N) content of shoots was similar among low, medium and high P doses in inorganic P source. However, in organic P source, N content in shoots was increased as higher P was applied (Fig. 4c, Table 1). In addition, the presence or absence of AMF in roots altered total N in shoots of tomato plants via genotype interaction with P sources, where shoot N content was higher in 76R (mycorrhizal) plants

**Table 1**
Three-way ANOVA summary table for plant and soil variables at the time of harvest. Factors in the analysis were P dose (P), P source ratio (R) and mycorrhizal genotype (G). Both main effects and interaction terms are indicated: "*" $P < 0.05$; "**" $P < 0.01$; "***" $P < 0.001$.

| | G:P:R | G:R | P:G | P:R | G | P | R |
|---|---|---|---|---|---|---|---|
| Plant-available soil P | ns | ns | ns | *** | ns | *** | *** |
| Shoot dry weight | ns | *** | ns | *** | ** | *** | *** |
| Root dry weight | ns | ns | ns | *** | ns | *** | *** |
| Shoot P content | ns | *** | ns | ns | ns | *** | ** |
| Root P content | ns | ns | ns | * | ns | *** | *** |
| Shoot N content | ns | *** | ns | *** | ns | *** | *** |

than in *rmc* (non-mycorrhizal) plants in the (1:0) treatments while the reverse trend was true in the (0:1) treatments, and no difference was found in the (1:1) treatments.

### 3.4. Mycorrhizal colonisation, growth and P responses

Whereas there was no AM colonisation formed in roots of tomato *rmc* genotype, mean percent AM colonisation of tomato 76R genotype ranged from 7% to 38.2% (Fig. 3b). Mycorrhizal colonisation was not different between low and medium P doses at 10 and 20 mg $kg^{-1}$. However, AM colonisation was decreased by half when application rate of P increased to 40 mg $kg^{-1}$. Plant AM colonisation was not affected by P sources at any of the P dose treatments (Table 2).

Whereas P source and dose did not affect MGR and MPR of plants (sum of shoots and roots), they affected MGR and MPR of shoots (Fig. 5a–b, Table 2). Specifically, MGR of shoots were positive in the inorganic P sources at all rates of P doses, and negative in organic P source at high P rate (Fig. 5a). Similarly, MPR of shoots were positive, neutral and negative as soils were applied with inorganic P source, mixed P source and organic P source, respectively (Fig. 5b, Table 2).

**Table 2**
Two-way ANOVA summary table for mycorrhizal colonisation and responses. Factors in the analysis were P dose (P) and P source ratio (R). Both main effects and interaction terms are indicated: "*" $P < 0.05$; "**" $P < 0.01$; "***" $P < 0.001$.

| | P:R | P | R |
|---|---|---|---|
| % Colonisation | ns | *** | ns |
| % Shoot_MGR | * | ns | *** |
| % Shoot_MPR | ns | ns | *** |

## 4. Discussion

The addition of different sources of P, at a range of application rates to the soil had a very clear impact on the growth, nutrition and mycorrhizal colonisation of tomato roots in this experiment. The results indicated that even when plants were supplied with the same amount of total P, the form in which it was supplied had an impact on plant growth and P nutrition. Interestingly, mycorrhizal growth and P responses varied considerably with the sources of P applied, regardless P application rate.

### 4.1. Effects of P source and P dose on mycorrhizal colonisation

Mycorrhizal colonisation, as expected, was reduced at high soil P availabilities (Watts-Williams et al., 2018). However, in this experiment, the reduction was not observed between low and medium P doses, which suggested that mycorrhizal colonisation of roots may not exhibit negative linear relationship with soil P up until a critical level. This argument is supported by previous research in crop plants and multipurpose tree species where the application of P to soils up to 20 mg $kg^{-1}$ did not adversely affect mycorrhizal colonisation (Shukla et al., 2012). In addition, the absence of the P source effect on mycorrhizal colonisation of roots is perhaps not surprising given that P dose treatments were below the critical threshold of 20 mg $kg^{-1}$ at low and

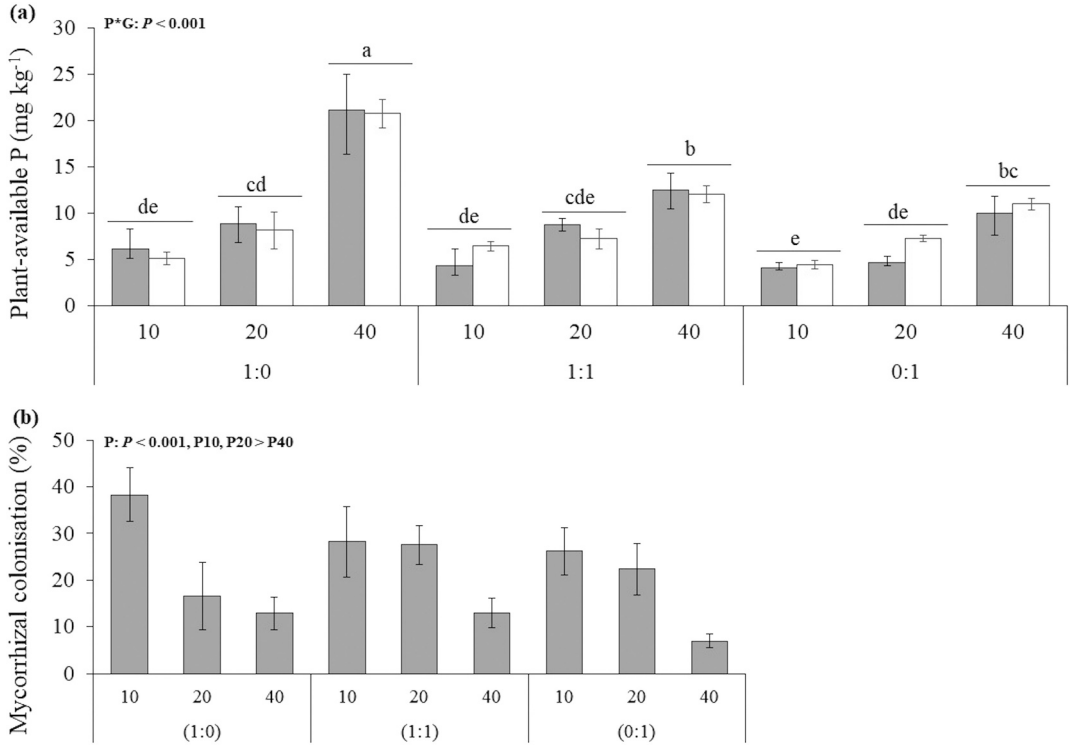


**Fig. 3.** Plant-available P concentration in soil at 42 DAP that hosted 76R (mycorrhizal) plants (grey bars) and *rmc* (non-mycorrhizal) plants (white bars) (a) and mycorrhizal colonisation of 76R (mycorrhizal) plants (b) with P source ratios of (1:0), (1:1) and (0:1) and at soil P doses of 10, 20 and 40 mg $kg^{-1}$. Values are mean ± SEM, $n = 5$. Significant interacting and main effects are indicated in figures. Means with at least one common letter are not significantly different at the $P < 0.05$ level. Lines above bars indicate two-way interacting effects of P source ratio and P dose.

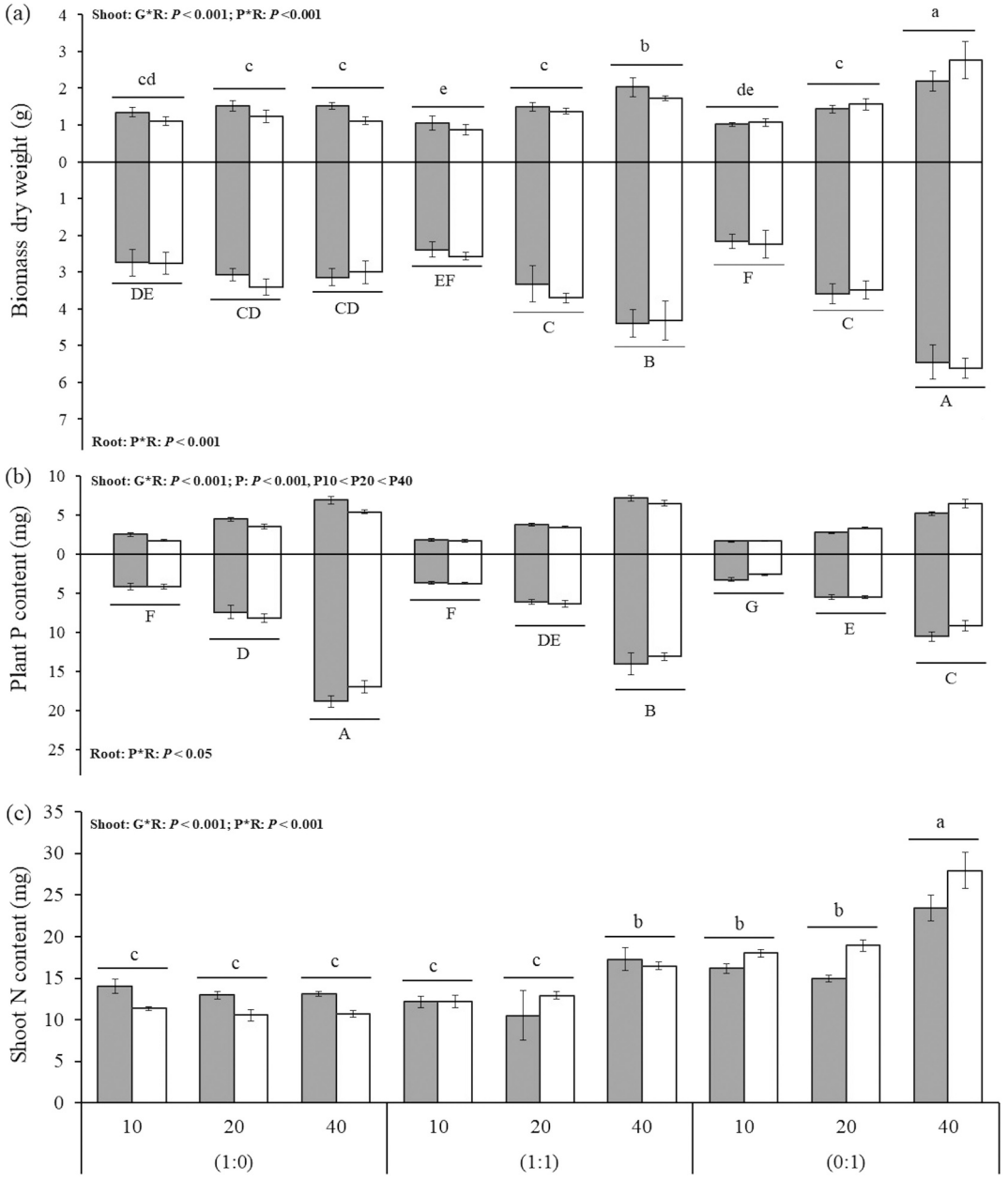


**Fig. 4.** Biomass dry weight (a), total P content (b) and total N content (c) in shoot (above horizontal axis) and root (below horizontal axis) of 76R (mycorrhizal) plants (grey bars) and *rmc* (non-mycorrhizal) plants (white bars). Plants were grown with P source ratios of (1:0), (1:1) and (0:1) and at soil P doses of 10, 20 and 40 mg $kg^{-1}$. Values are mean ± SEM, $n = 5$. Significant interacting and main effects are indicated in figures. Means with at least one common letter are not significantly different at the $P < 0.05$ level. Lines above bars indicate two-way interacting effects of P source ratio and P dose.

medium P application rates.

### 4.2. Contrasting mycorrhizal growth and P responses to inorganic and organic P sources and doses

Our results show the important role of shoot P content on the way plants responded to AMF; shoot P content made the greatest contribution to PC1, with shoot dry weight having a similar impact. It is widely suggested that P concentration of plant tissues and cells regulate mycorrhizal growth and P responses of plants; that is, plants with higher P concentration have a reduced dependence on the mycorrhizal pathway of P uptake (Breuillin et al., 2010). However, this was not the case in inorganic P type treatments where the mycorrhizal growth response remained positive while plant P concentration continued to increase from deficient to sufficient (Reuter and Robinson, 1997). A greater shoot P content in higher P doses was explained as the result of an increase in plant-available P in the soil following the application of the inorganic P source. In the inorganic P source treatment, tomato plants relied heavily of AMF for P acquisition in all P doses, as indicated by positive shoot MPR. Compared to the lower P dose at 10 and 20 mg $kg^{-1}$, the application of P at 40 mg $kg^{-1}$ resulted in lower AM colonisation; however, shoot MGR was greater, likely because the extent of root colonised by AMF is not necessarily correlated with the effects of colonisation on biomass and P nutrition (Feldmann et al., 2009). In addition, the trend towards greater MGR at high P dose may suggest that the cost of C—P trade-off was lower than with the lower P additions, given that the lower number of exchanged C—P sites was formed due to lower AM colonisation while AMF contribute equal amount of P to plant under high and low P addition (Thingstrup et al., 2000).

In contrast to the inorganic P source, AM associations had negative

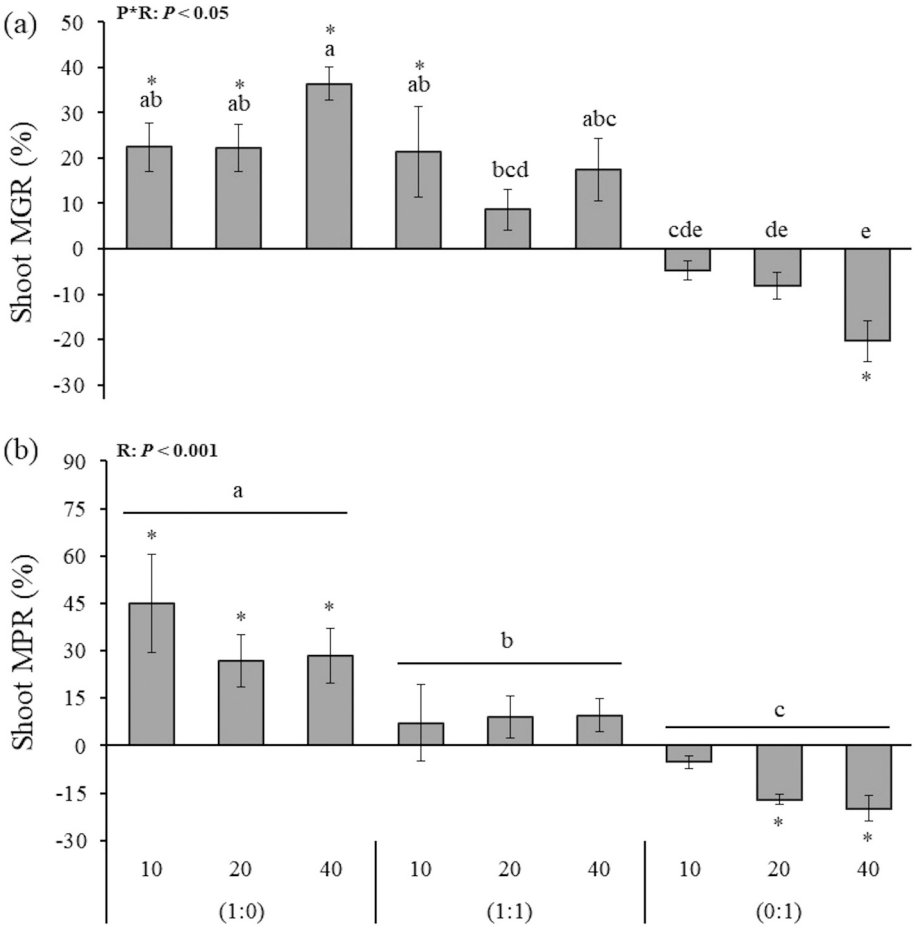


**Fig. 5.** Mycorrhizal growth response (MGR) (a) and mycorrhizal P response (MPR) (b) of shoots in 76R (mycorrhizal) plants grown with P source ratios of (1:0), (1:1) and (0:1) and at soil P doses of 10, 20 and 40 mg $kg^{-1}$. Values are mean ± SEM, $n = 5$. Significant interacting and main effects are indicated in figures. Means with at least one common letter are not significantly different at the $P < 0.05$ level. Lines above bars indicate the main effect of P source ratio. Asterisks indicate means that are different from 0 at 95% CI.

effects on shoot growth (MGR) and shoot P nutrition (MPR) when plants were supplied with chicken litter only. As the direct (root) P uptake pathway of plants can be down-regulated in mycorrhizal colonised roots (Watts-Williams et al., 2015), the lower total P uptake in mycorrhizal plants may be explained by low P uptake by external hyphae in the organic P source (Feng et al., 2003) which may not be compensated by root (direct pathway) uptake. In addition, applying chicken litter may stimulate external AM hyphal growth (Aleklett and Wallander, 2012; Hammer et al., 2011) to enhance mineralisation of organic P source (Jiang et al., 2021). Thus, the increase in hyphal growth may enhance C drain to AMF, causing a reduction in shoot growth in the mycorrhizal plants compared to the non-mycorrhizal plants. However, discovering the precise effects of the mycorrhizal pathway in the inorganic or organic P treatments, may require further investigation, involving a combination of physiological, isotopic and molecular approaches.

### 4.3. Neutral mycorrhizal growth response in the mixed P source

In addition to adding P as purely inorganic or organic fertiliser sources, it was also added as a mixture of the two. We found that the combined use of inorganic and organic P sources helped to improve available soil P and plant P uptake compared to solely organic P source. In comparison with the inorganic P source, the mixed P source increased plant growth while plant P uptake was closely matched. Thus, it is likely that the inorganic P source provided an initial pulse of P to support early plant growth and development, and the organic source then provided a sustained P supply (via mineralisation) over the course of the plant growth cycle. Accordingly, the combined use of inorganic and organic P sources may be one means to reduce the supply of inorganic P source at the beginning of growing season, while maintaining an adequate supply to match later demand for P. The combined use of P sources lessened the adverse effects of AMF on tomato shoot growth compared to organic P sources. Given that the combined use provided different proportions of P and N, compared to the solely inorganic P, and the solely organic material, treatments, the changes in mycorrhizal growth and P nutrition responses may be due to this shift in P and N balance (Aleklett and Wallander, 2012; Hoeksema et al., 2010; van der Heijden and Kuyper, 2001). Thus, the results have shown context dependent responses of mycorrhizal plants; the mechanisms underlying the effects are worthy of further investigation.

## 5. Conclusions

Our research has shown that the inputs of P to the soil were correlated with available soil P and plant P uptake; whereas the inorganic P source provided higher readily available P compared to organic P source, the combined use of those two materials provided intermediate results. The research also highlighted that P application rate but not P source affected mycorrhizal colonisation. However, mycorrhizal growth and P responses changed along a continuum from positive to negative depending on P source. Subsequently, clear changes in mycorrhizal growth and P responses at the same application rates of P provided strong context dependency of mycorrhizal responses. While the blending of different P sources could be used to manipulate mycorrhizal symbiosis to enhance plant growth and P nutrition, this study opens new alternatives in the formulation of P fertilisers in combination with AMF for practical application.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Supplementary

Table S2. Characteristics of the soils at the time of planting after incorporating inorganic P and organic material (chicken litter). The P ratio treatment refers to the proportion of total P supplied as phosphoric acid relative to chicken litter. Values are mean ± SE, *n* = 10. Within column, means with at least one common letter are not significantly different at the P <0.05 level.

| P ratio | Total P applied (mg $kg^{-1}$) | Plant-available P (mg $kg^{-1}$) | pH | EC (µS/cm) | $NH_4NO_3$ (mg $kg^{-1}$) |
|---|---|---|---|---|---|
| (1:0) | 10 | 11.87±1.51(de) | 5.68±0.06(cd) | 67.53±12.46(b) | 20.82±1.55(d) |
| | 20 | 18.95±1.26(bc) | 5.52±0.03(de) | 56.90±1.22(b) | 19.52±0.70(d) |
| | 40 | 34.78±2.75(a) | 5.39±0.04(e) | 56.07±1.76(b) | 19.52±1.15(cd) |
| (1:1) | 10 | 9.37±1.30(def) | 5.67±0.03(cd) | 61.03±3.18(b) | 23.31±1.14(bcd) |
| | 20 | 14.59±0.50(cd) | 5.82±0.05(c) | 74.83±7.1(ab) | 25.06±0.87(bc) |
| | 40 | 22.69±1.23(b) | 5.90±0.04(bc) | 76.60±1.42(ab) | 25.83±0.69(b) |
| (0:1) | 10 | 5.42±0.39(f) | 5.81±0.04(c) | 65.07±0.42(b) | 25.56±0.95(bc) |
| | 20 | 7.90±0.47(ef) | 6.14±0.07(ab) | 76.33±1.92(ab) | 27.70±0.82(b) |
| | 40 | 13.99±1.29(cde) | 6.27±0.02(a) | 93.87±1.09(a) | 36.00±1.63(a) |

# CHAPTER 4: COMBINING ORGANIC AND INORGANIC SOURCES OF PHOSPHORUS FERTILISER SUSTAINS TOMATO GROWTH OVER TIME.

# Statement of Authorship

| | |
|---|---|
| Title of Paper | Combining organic and inorganic sources of phosphorus fertiliser sustains tomato growth over time. |
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| Contribution to the Paper | Contributed to the development of ideas, performed glasshouse and laboratory work, interpreted data and wrote the manuscript. | | |
| Overall percentage (%) | 85% | | |
| 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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## 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.

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**Combining organic and inorganic sources of phosphorus fertiliser sustains tomato growth over time.**

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## Highlight

High-throughput shoot phenotyping technology demonstrated that there were early benefits of arbuscular mycorrhizal colonisation on tomato shoot growth that became neutral over time in various soil N and P concentrations.


## Abstract

While the application of nitrogen (N) and phosphorus (P) to soils is required to maintain plant yields, arbuscular mycorrhizal (AM) colonisation of roots can improve plant nutrient acquisition. We quantified temporal growth responses of tomato plants with low and high rates of N and P application using a high-throughput shoot phenotyping system. Organic, inorganic and mixed sources of P were incorporated into the soil to grow a mycorrhiza-defective mutant tomato, along with its wildtype progenitor. At the same rate of total P application, the inorganic P source resulted in faster shoot growth (imaged projected shoot area) at early time points. Later on, the plants supplied with organic or mixed P sources grew faster than those that received the inorganic P source, resulting in comparable shoot biomass in all treatments at the time of destructive harvest. The AM plants achieved maximum shoot growth slightly earlier than non-AM plants. Our results indicated that the combined use of inorganic and organic P sources, as well as forming AM associations, produced rapid and high shoot growth compared to the other treatments. The results also demonstrated that without daily shoot phenotyping, single endpoint analysis of shoot biomass can lead to different interpretations of treatment effects (fertiliser, AM fungi) on the plants.



**Keywords:** Arbuscular mycorrhizal (AM) fungi, fertiliser, high-throughput phenotyping, organic material, phosphorus, tomato

## 1. Introduction

Phosphorus (P) fertilisers are applied to soils to increase crop yields (Hopkins and Hansen, 2019) as P – a major cellular constituent (e.g. of nucleic acids, phospholipid) – is the nutrient that most limits plant production globally, after nitrogen (N) (Hawkesford *et al.*, 2012). However as inorganic P fertilisers are derived from finite phosphate rock deposits (Ashley *et al.*, 2011), the use of inorganic fertilisers is becoming more expensive and less accessible, especially to growers with low incomes or limited access to supply chains (Alewell *et al.*, 2020). In contrast, P-rich organic wastes such as crop residues and animal manures are produced in large quantities globally and are readily available to many farmers who do not have access to mineral fertilisers (Powers *et al.*, 2019). Thus, there is a potential to use P-rich organic materials to reduce reliance on inorganic P fertiliser inputs, and to make use of materials that might otherwise go to waste. However, repeated application of P fertilisers can lead to a build-up of P in the soil (Lopez-Arredondo *et al.*, 2014), which may result in pollution and eutrophication (Scavia *et al.*, 2014). The accumulation of P occurs in both conventional (Bouwman *et al.*, 2017) and organic (Cooper *et al.*, 2018) farming systems. This could be because soluble P forms are less accessible to plant roots following fertiliser application due to adsorption and precipitation reactions (McLaughlin *et al.*, 2011), or because application rates of manures are high (Qaswar *et al.*, 2020). Thus, managing P application to increase plant P acquisition without unwanted environmental impacts is an important goal.

The uptake of P as orthophosphate occurs by plant transporters at the root surface (Magalhaes *et al.*, 2017). The acquisition of P by roots is limited by the concentration of orthophosphate in soil solution and the chance for roots to encounter P in the soil (Shen *et al.*, 2011). In comparison with organic P sources, inorganic P sources contain higher concentrations of orthophosphate, thus they are more plant-available (Azevedo *et al.*, 2018). In addition, much of the P in organic materials is taken up by plants relatively slowly, because P in organic forms needs to be mineralised before it is plant available (Bünemann, 2015; Dey *et al.*, 2019). It has been suggested that the co-application of organic materials with inorganic P sources may help supply P in a manner that improves plant performance (Timsina, 2018). Plants have evolved a number of mechanisms by which they can improve P acquisition, such as root proliferation in soil P hotspots, increasing the frequency and length of root hairs, forming root clusters, and modifying root architecture towards more efficient P uptake (Funayama-Noguchi *et al.*, 2015; Holford, 1997; Lambers *et al.*, 2008). In addition, association with arbuscular mycorrhizal (AM) fungi is another mechanism for improving plant P uptake (Rillig *et al.*, 2016). For example, external hyphae of AM fungi can grow up to 30 mm from root surfaces, acquiring distant P, and increasing P transport (to the plant) up to 15-fold (Mai *et al.*, 2019). Arbuscular mycorrhizal fungi also have high-affinity P transporter proteins that enable the transport of P into their hyphae at lower soil P concentrations compared to plant roots (Xie *et*

*al.*, 2016). Moreover, AM hyphae promote mineralisation of organic P materials by excreting phosphatases (Prasad *et al.*, 2012) and interacting with and assisting P-solubilising bacteria (Jiang *et al.*, 2020; Xu *et al.*, 2018). As most crop plant species form AM associations (Chen *et al.*, 2018), colonisation of roots by AM fungi can help plants to improve P acquisition, especially in low available soil P conditions. Despite that, the formation of AM associations has been shown to result in positive, neutral or negative impacts on plant growth, depending on soil nutrients and plant types (Hoeksema *et al.*, 2010). In addition, the effects of forming AM associations on plant growth may change over plant's life, as plants reduce dependence on AMF when their roots develop (length, weight and density) (Tawaraya, 2003). Thus, it may be important to understand how different plant growth stages are affected by AMF.

Plants can change their growth and nutritional responses to AM fungi over their lives (Ronsheim, 2012; Watts-Williams et al., 2019) and depending on environmental conditions such as soil P availability (Bonneau et al., 2013; Yang et al., 2016). In addition to soil P, a change in soil N availability affects not only plant growth but also P nutrition and AM responses (Ingraffia et al., 2020; Riley et al., 2019; Sylvia and Neal, 1990; van der Heijden and Kuyper, 2001). Here we aimed to explore temporal responses of AM and non-AM tomato plants, to three sources and two rates of P (organic and/or inorganic), and two rates of N, using a high-throughput shoot phenotyping system (Berger et al., 2012; Riley et al., 2019). Arbuscular mycorrhizal colonisation treatments were established using an AM-defective tomato mutant and its AM wild-type progenitor (Watts-Williams and Cavagnaro, 2014), so that AM effects could be studied without the confounding effects of soil sterilisation on P mineralisation and the wider soil microbiome. Specifically, we hypothesised that:

i. Plant growth will be greater in response to inorganic P compared to organic P material due to the higher starting available P concentration;
ii. An effect of organic P material on shoot growth will appear later than that of inorganic P; and
iii. Arbuscular mycorrhizal fungi will have more pronounced effects on plant growth during earlier, rather than later, stages of shoot growth.

## 2. Materials and Methods

### *2.1. Soil, mycorrhizal and non-mycorrhizal tomato genotypes*

The soil used in this experiment was a sandy loam collected from the Waite Arboretum, South Australia (S34°58'01'', E138°37'46'') which was air-dried, sieved, mixed and stored in closed containers. Prior to use, soil was mixed with dry fine sand (1:9 *w/w*, referred to hereafter as "soil") to reduce plant-available P of the soil (bicarbonate-extractable P = 4.17 mg P $kg^{-1}$) and to facilitate

root sampling at the end of the experiment. The experiment used 1.2 L free-draining pots, containing 1,330 g soil and 70 g inoculum soil of the AMF, *Rhizophagus irregularis* (DAOM181602). In an effort to re-establish the soil microbiota and to minimise potential impacts of re-wetting soil before planting (Gao *et al.*, 2020), the soil was pre-incubated at room temperature with 70 mL RO water (5 % *w/w*) soil water content) for three weeks prior to planting.

The experiment used two indeterminate tomato (*Solanum lycopersicum* L.) genotypes that contrast in their ability to form AM associations. The mycorrhiza-defective mutant (*rmc:* -AM) and its wild-type progenitor (76R: +AM) have been used extensively to study the impacts of AM associations on plant growth and nutrition (Watts-Williams and Cavagnaro, 2015). Briefly, the tomato 76R variety has been found to be coloniseed by the *R. irregularis* isolate (DAOM181602) whereas the tomato *rmc* variety was not (Gao *et al.*, 2001; Manjarrez *et al.*, 2008). In addition, the 76R/*rmc* tomato pair are matched in terms of growth in the absence of AMF (Watts-Williams and Cavagnaro, 2015). Seeds of both genotypes were surface-sterilised by immersion in 70 % ethanol and 4 % NaOCl for 15 minutes, rinsed with RO water, and germinated in sand for three weeks to produce seedlings (first true leaf stage) before being transplanted into the prepared pots.

### *2.2. Nutrient treatments*

Phosphorus was applied to the soil at two rates: 10 (LP) and 30 (HP) mg P $kg^{-1}$ soil as one of three P sources: P-rich organic material alone (OM-P; dry and un-ground chicken litter), inorganic P source alone (IN-P; phosphoric acid) and OM/IN-P source (1:1 mixed OM-P:IN-P ratio, mg P/mg P). Chicken litter used in this experiment was from the same batch that had been characterised previously (Mackay *et al.*, 2017) and had 9.9 C/N ratio, 38.7 g N $kg^{-1}$ and 16.5 g P $kg^{-1}$. Chicken litter was weighed for each individual pot following corresponding P rate and P type. Inorganic P material was prepared from phosphoric acid (following Bertrand *et al.* (2006)). Specifically, 7.9 mL of 85% phosphoric acid (density = 1.685 g/$cm^3$) was diluted in 500 mL reverse osmosis (RO) water that provided 42 mg P $pot^{-1}$ in a 5 mL aliquot (equal to 30 mg P $kg^{-1}$ soil). The diluted phosphoric acid solution was further diluted with RO water to make up 5, 10 and15 mg P $kg^{-1}$ soil in a 5 mL volume. Phosphoric acid was used as the inorganic P source as it only supplies P nutrient to a soil with minimal effect on soil pH (Mackay *et al.*, 2017). Nitrogen was applied as $NH_4NO_3$ solution on the soil surface of pots at two application rates: 17.5 (LN) and 70 (HN) mg N $kg^{-1}$ soil.

Immediately prior to planting, OM-P and IN-P treatments were added to the soil by mixing these materials with soil in plastic bags, by hand for one minute. For the IN-P treatment, each pot received 5 mL pre-prepared diluted phosphoric acid, 45 mL RO water and 20 mL modified Long-Ashton mineral solution (N included and P omitted). For the OM-P treatment, each pot received pre-weighed chicken litter, 50 mL RO water and 20 mL modified Long-Ashton mineral solution. For

the OM/IN treatment, each pot received 5 mL previous prepared diluted phosphoric acid, pre-weighed chicken litter, 45 mL RO water and 20 mL modified Long-Ashton mineral solution. Soil sub-samples (50 g) were taken from each bag to quantify plant-available P (bicarbonate-extractable P), electrical conductivity (EC), pH and available N at the time of planting (Table S2). Soil was packed into the pots at 10 % moisture content (*w/w*). On the day of planting (0 DAP), one tomato seedling was transplanted into each pot. Nitrogen was added to pots once on 13 DAP, by dispensing either the previously prepared LN or HN solutions on soil surface prior to watering.

The experiment was comprised $2 \times 2 \times 3 = 12$ nutrient treatment groups, corresponding to two soil N addition rates, two soil P addition rates, and three P sources. The nutrient treatment groups were grown with the two tomato genotypes, making $2 \times 12 = 24$ treatments for the whole experiment. With five replicates for each treatment combination, there was a total of $n = 120$ plants. Each replicate occupied two lanes × 12 positions in the NE Smarthouse of The Plant Accelerator (see below), for a total of 10 lanes × 12 positions. Randomisation was based on a latinised resolved row-column design generated using the *od* (Butler, 2018), and *dae* (Brien, 2020b) packages for the R statistical computing environment (R Core Team, 2020).

### *2.3. High-throughput shoot phenotyping and plant management*

The experiment was conducted in June - July (Austral winter) in the NE Smarthouse of The Plant Accelerator, Australian Plant Phenomics Facility, located at the University of Adelaide, Waite Campus, Australia. Plants were grown on a bench until 7 DAP, after which they were loaded onto the conveyor system of the high-throughput shoot phenotyping (HTP) facility, until 42 DAP (Brien *et al.*, 2013). With this automatic system, shoot growth was imaged daily using the Scanalyzer 3D imaging system (LemnaTec GmbH, Aachen, Germany) (Berger *et al.*, 2012). Red-green-blue (RGB) images were taken from three views, comprising two side views at an angular separation as close as practicable to 90 degrees and a view from above. Plants were watered to 10 % (*w/w*) soil mass by the automated system, on a daily basis. A modified Long-Ashton mineral solution (N included and P omitted) (following Cavagnaro *et al.* (2001)) was supplied to the plants at a rate of 20 mL $pot^{-1}$ on 6, 20, 27, and 34 DAP. During the experiment, the ambient temperature was maintained at an average of 24°C/17°C day/night cycle. The average light levels at midday were 280 µmol $m^{-2}$ $s^{-1}$ and an average nine hour day length, which was adequate for growing tomato plants in winter (Tartachnyk and Blanke, 2007).

The imaging data was prepared using the SET method (Brien *et al.*, 2020) employing the *growthPheno* package (Brien, 2020c) with the R statistical computing environment (R Core Team, 2020). The Projected Shoot Area (PSA) of the plant was defined as the sum of the areas (kilopixels) as measured from three RGB images. The raw data for DAP 34 were removed from the data set

because the plants were noticeably water-stressed. Spline smoothing was applied to the PSA curve of each plant to remove transient fluctuations in the trend over time, yielding smoothed projected shoot area (sPSA). Data smoothed using different degrees of freedom were compared by using *probeSmoothing* from *growthPheno,* after which six degrees of freedom (*df* = 6, mild smoothing) was chosen subjectively as appropriate for smoothing this dataset and sPSA obtained. Then the smoothed absolute growth rate (sPSA AGR, kilopixels/day) describes the estimated daily rate of accumulation of shoot biomass and was calculated based on the sPSA data. In particular, the sPSA AGR from DAP t1 to t2 is given by Eqn.1, where sPSA1 and sPSA2 are the projected shoot areas at t1 and t2, respectively:

$$\text{sPSA AGR} = \frac{\text{sPSA2} - \text{sPSA1}}{\text{t2} - \text{t1}} \qquad (1)$$

After that, to investigate the growth dynamics, the smoothed data was used to produce single-day responses sPSA at DAP 13, 16, 19, 22, 25, 30, 36 and 42; and interval responses for sPSA AGR and at 13–16, 16–19, 19–22, 22–25, 25–30, 30–36 and 36–42.

To produce phenotypic predictions, or adjusted means, a fixed-model analysis was performed for each imaging or harvest trait using *ASReml-R* (Butler et al., 2020) and *asremlPlus* (Brien, 2020a) packages with the R statistical computing environment (R Core Team, 2020). The model for this analysis is of the form (Eqn.2)

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{e} \qquad (2)$$

where $\mathbf{y}$ is the response vector of values for the trait being analysed; $\boldsymbol{\beta}$ is the vector of effects, $\mathbf{e}$ is the vector of residual effects, and $\mathbf{X}$ is the design matrix corresponding to $\boldsymbol{\beta}$. The effect vector $\boldsymbol{\beta}$ is partitioned as

$$\boldsymbol{\beta}^{\top} = [\mu\ \boldsymbol{\beta}_{\mathbf{R}}^{\top}\ \beta_{\text{xPosn}}\ \boldsymbol{\beta}_{\text{G}}^{\top}\ \boldsymbol{\beta}_{\text{N}}^{\top}\ \boldsymbol{\beta}_{\text{P}}^{\top}\ \boldsymbol{\beta}_{\text{S}}^{\top}\ \boldsymbol{\beta}_{\text{G:N}}^{\top}\ \boldsymbol{\beta}_{\text{G:P}}^{\top}\ \boldsymbol{\beta}_{\text{G:S}}^{\top}\ \boldsymbol{\beta}_{\text{N:P}}^{\top}\ \boldsymbol{\beta}_{\text{N:S}}^{\top}\ \boldsymbol{\beta}_{\text{P:S}}^{\top}\ \boldsymbol{\beta}_{\text{G:N:P}}^{\top}\ \boldsymbol{\beta}_{\text{G:N:S}}^{\top}\ \boldsymbol{\beta}_{\text{G:P:S}}^{\top}\ \boldsymbol{\beta}_{\text{N:P:S}}^{\top}\ \boldsymbol{\beta}_{\text{G:N:P:S}}^{\top}],$$

where $\mu$ is the overall mean and the $\boldsymbol{\beta}$ sub-vectors correspond to the respective effects of replicates (R); east-west trend by position within the Smarthouse; main effects of the treatment factors genotype (G), N rate (N), P rate (P) and P source (S); two-way treatment interactions (G:N, G:P, G:S, N:P, N:S and P:S); three-way interactions (G:N:P, G:N:S, G:P:S and N:P:S) and four-way interaction (G:N:P:S). Thus, the first two $\boldsymbol{\beta}$ subvectors capture spatial variation within the Smarthouse, while the remaining subvectors capture treatment effects. The residual effects $\mathbf{e}$ were assumed to be normally distributed with variance $\sigma^2$, except that for some traits (shoot/root fresh/dry weight, AM colonisation and shoot P content) the variance was allowed to differ between combinations of N

and P. All residual plots were satisfactory, indicating that the four-way interaction model appeared to be appropriate. Next, for each trait, Wald F-statistics were produced for the treatment main effects and interactions, and these were used to identify a chosen model based on the statistically significant terms. Phenotypic predictions conforming to the chosen model were then obtained for each combination of tomato genotype, N addition rate, P addition rate and P source. Finally, least significant differences [LSD (5%)] were calculated for comparing pairs of predictions within a trait. In this way, models were selected and predictions obtained for all imaging and harvest traits.

Maximum growth rate (sPSA $AGR_{Max}$) and corresponding date (sPSA $AGR_{Max.DAP}$) were computed over all imaging days. In the case of the sPSA $AGR_{Max.DAP}$ trait, a modified version of the four-way interaction model was applied as the plants in the high N treatment had not yet achieved peak growth by the end of the imaging period. A nested model was used in which the effects of the other factor were examined within the two N rates. The vector $\boldsymbol{\beta}$ is now partitioned as $\left[\mu\ \boldsymbol{\beta}_{\mathbf{R}}^{\top}\ \beta_{\mathrm{xPosn}}\boldsymbol{\beta}_{\mathrm{N}}^{\top}\ \boldsymbol{\beta}_{\mathrm{G*P*S_{LN}}}^{\top}\ \boldsymbol{\beta}_{\mathrm{G*P_{HN}}}^{\top}\right]$ where $\boldsymbol{\beta}_{\mathrm{N}}^{\top}$ is the subvector of N main effects, $\boldsymbol{\beta}_{\mathrm{G*P*S_{LN}}}^{\top}$ is the subvector for the main effects, two interactions and three-way interaction for the factors G, P and S within the low N rate (LN) and $\boldsymbol{\beta}_{\mathrm{G*P_{HN}}}^{\top}$ is the subvector for the main effects and two interactions for the factors G and P within the high N rate (HN).

### *2.4. Harvesting, plant P analysis and AM colonisation*

On 42 DAP (when flowering had not started in all treatments), all tomato plants were destructively harvested. Shoots were cut at soil level and weighed, and then dried in an oven for a week at 60 °C and weighed again. The dry shoots were then ground to a fine powder using a puck mill pulveriser machine and analysed for total P concentration, following digestion in concentrated nitric acid and 36% hydrogen peroxide (1:4 *v/v*), P concentration was measured by inductively coupled plasma optical emission spectroscopy (ICP-OES) (following Wheal *et al.* (2011)). Roots were washed free of any attached soil with RO water, patted dry and weighed. Subsamples of ~200 mg fresh roots were randomly taken to represent different root depth and fixed by 50 % ethanol for 24 hours. Fixed roots 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 at 60 °C for 15 min (Vierheilig *et al.*, 1998), then de-stained in acidified water for 24 hours, before being stored in 50 % glycerol solution. Percent root length AM colonisation was estimated on stained root samples according to the gridline intersect method at 20 × magnification (Giovannetti and Mosse, 1980).

### *2.5. Calculations and statistical analyses*

Four-way ANOVA was performed on data for soil characteristics with four experimental factors: tomato genotype, N addition rate, P addition rate and P source, where appropriate. Where ANOVA interactions and/or main effects were significant ($P<0.05$), a Tukey's honestly significant difference (HSD) post hoc test was performed to identify differences between means among treatments with α level 0.05. Data analyses were performed in R (R Core Team, 2020).

## 3. Results

### *3.1. Phenotyping of shoot growth highlights the time-dependent response to P source and AM inoculation*

Prior to 36 DAP, sPSA (shoot growth) and sPSA AGR (shoot growth rate) were affected by the interaction of P source and rate, in which the separation of sPSA and sPSA AGR to different P sources were greater at HP than at LP (Fig.1, 2, Table S1). Plants that received the IN-P source had higher sPSA than the OM/IN-P and OM-P sources prior 36 DAP (Fig. 1). Similarly, plants grown with IN-P source had higher sPSA AGR than the plants grown with OM/IN-P and OM-P sources for the first four chosen time intervals (13-25 DAP) (Fig. 2). From 36 DAP, the sPSA and sPSA AGR were affected by the interaction of N rate and P source and/or P rate (Table S1). Specifically, the IN-P source had similar or higher sPSA compared to other P sources in the HN rate for 36 DAP onwards, and similar or lower in the LN rate for this late period (Fig. 1). In addition, in the last three time intervals (spanning 30 to 42 DAP), the sPSA AGR of the OM/IN-P and OM-P sources were similar or higher than IN-P source (Fig. 2), leading to greater sPSA $AGR_{max}$ value in the other sources compared to the inorganic P source, despite the later effect (Fig. 3).

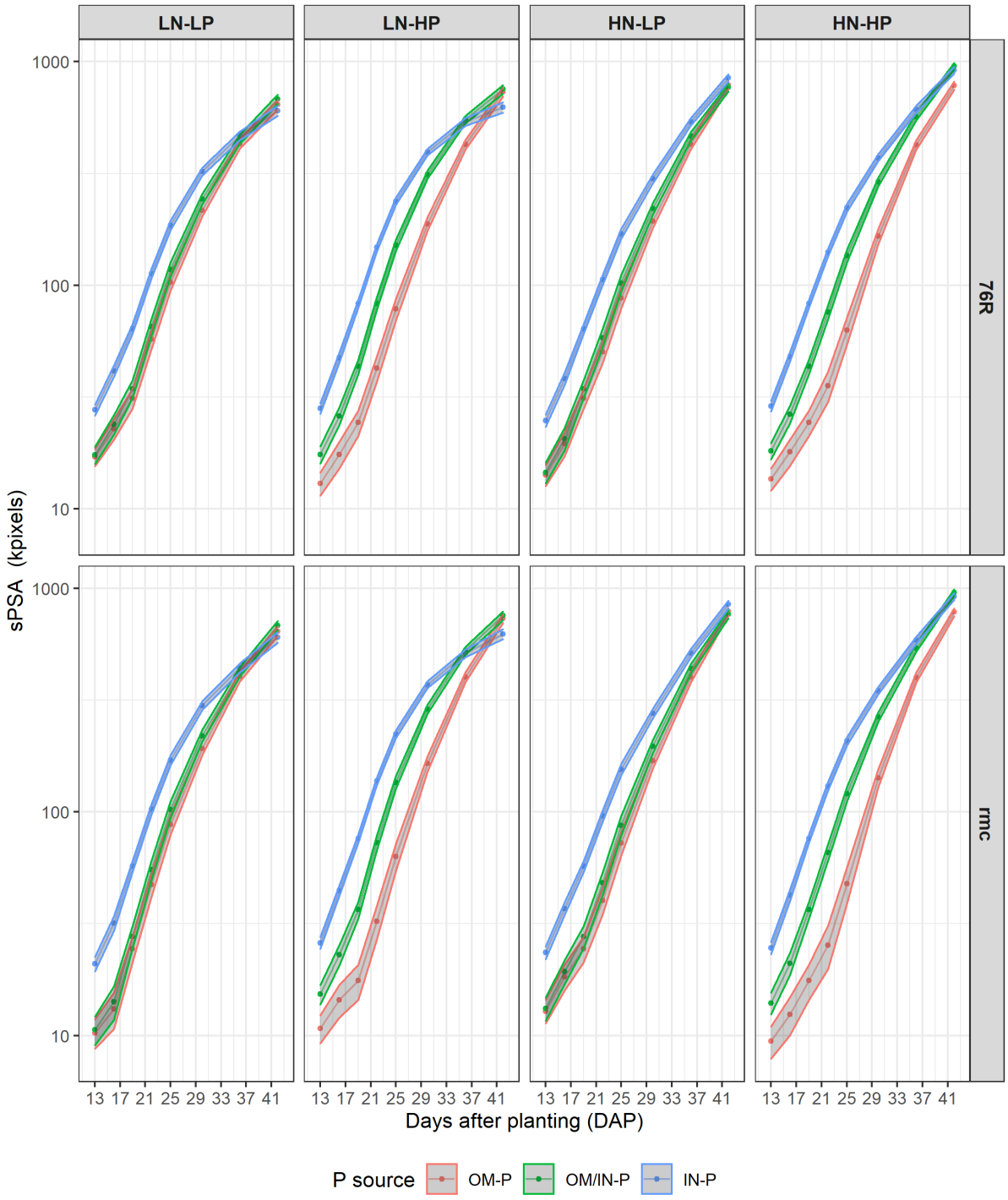


**Fig. 1.** Phenotypic predictions for the Smoothed Projected Shoot Area (sPSA) over DAP 13-42 plotted on a log scale for the three P sources OM-P, OM/IN-P and IN-P, grouped (i) horizontally by N rates (LN and HN) and P rates (LP and HP) and (ii) vertically by tomato genotype (76R (+AM) or rmc (-AM)). The width of the ribbons is equal to the least significant differences within a DAP. The predictions are not significantly different ($p > 0.05$) where the ribbons overlap.

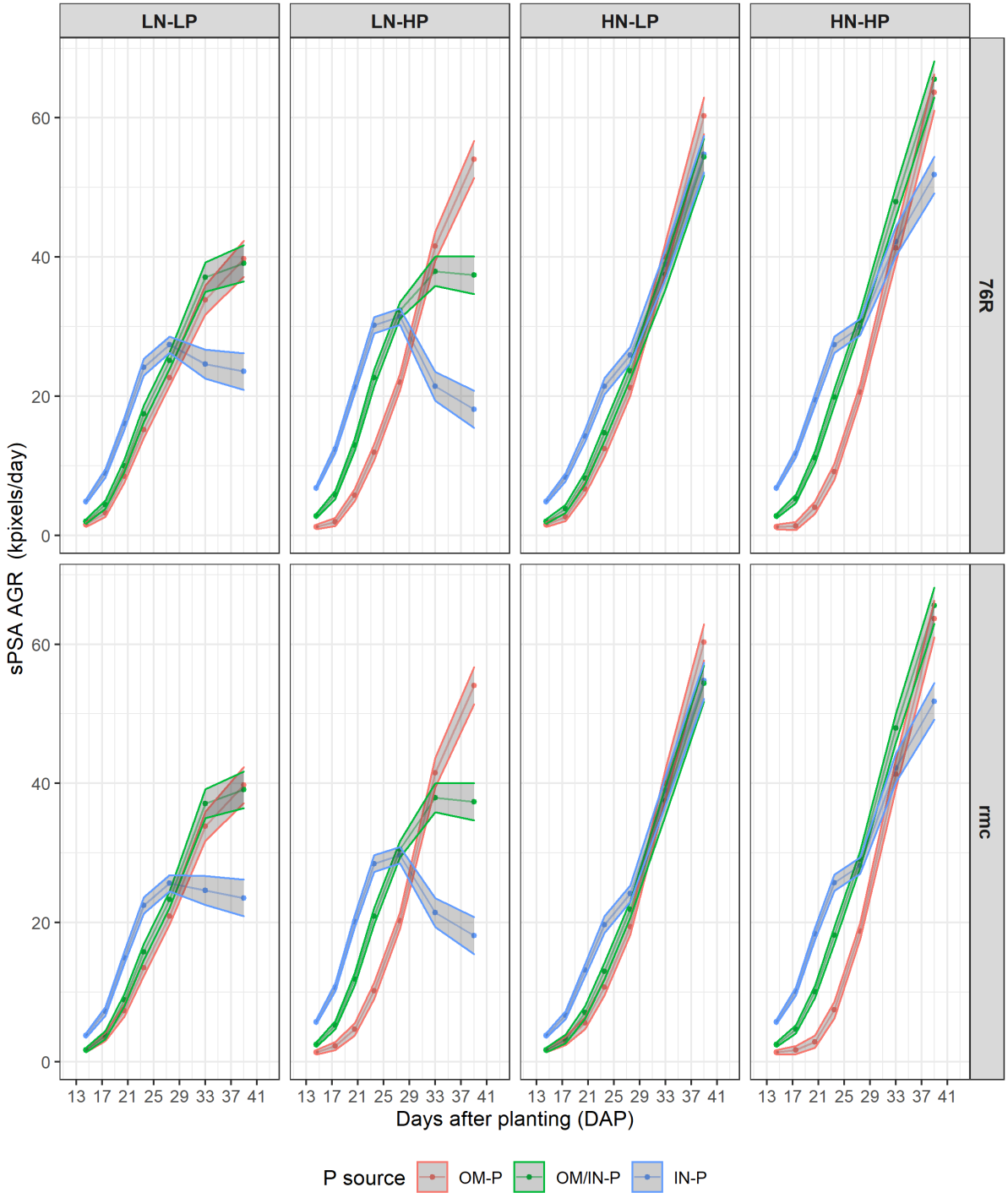


**Fig. 2.** Phenotypic predictions for the smoothed absolute growth rates (sPSA AGR) over DAP 13-42 for the three P sources: OM-P, OM/IN-P and IN-P, grouped (i) horizontally by N rates (LN and HN) and P rates (LP and HP) and (ii) vertically by tomato genotype (76R (+AM) or rmc (-AM)). The width of the ribbons is equal to the least significant differences within a DAP. The predictions are not significantly different (*p* > 0.05) where the ribbons overlap.

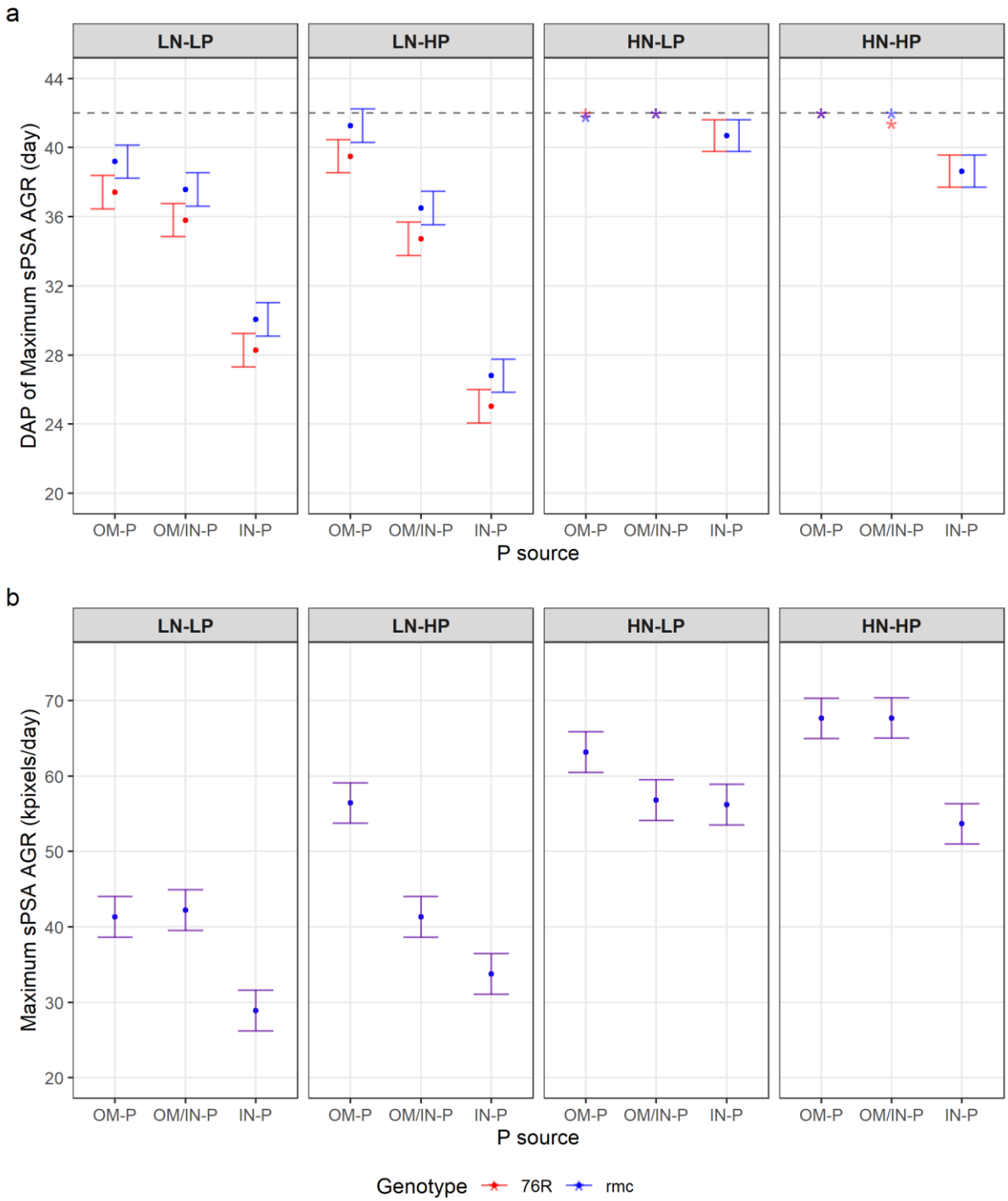


**Fig. 3**. Phenotypic predictions for the maximum growth rate (a) and the corresponding DAP (b) for tomato 76R (+AM) and *rmc* (-AM) genotypes grown using three P sources (OM-P, OM/IN-P and IN-P) and grouped by N rates (LN and HN) and P rates (LP and HP). Error bars correspond to half of the least significant difference [±half-LSD (5%)]. Error bars that overlap indicate that the predictions are not significantly different. The asterisks ("*") indicate that the maximum sPSA AGR occurred at the end of imaging (DAP 41-42) for all plants and without sPSA AGR having peaked and so the DAP mean is presented (the dashed line marks DAP 42). N.B. Predicted means and error bars of 76R and *rmc* are overlapped in (b).

The effects of AM colonisation on plant growth traits appeared early on in the experiment (Table S1). Examination of the predictions showed, for example, the +AM plants had greater sPSA

values than the -AM plants in the LN/LP and HN/HP treatments at 13 DAP and 16 DAP (Fig. 4). Similarly, +AM plants had a higher sPSA AGR than -AM plants for the first two intervals (until 19 DAP) in the IN-P treatment (Fig. 5). Despite the positive response to AM colonisation early, both shoot biomass and growth rate responded in a neutral manner to AM colonisation from 19 DAP onwards, in all treatments (Fig S1, Fig. S2).

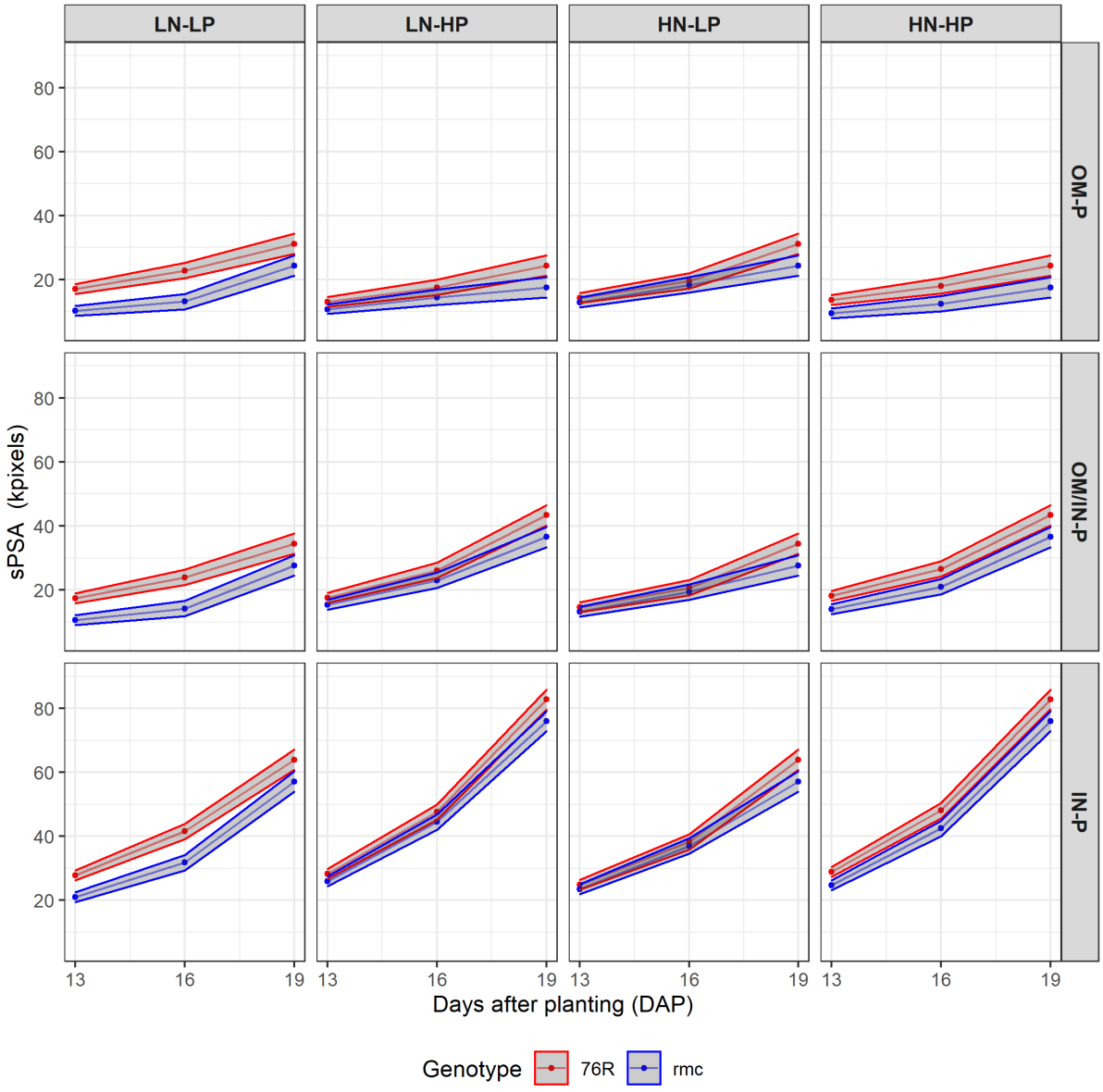


**Fig. 4.** Phenotypic predictions for the Smoothed Projected Shoot Area (sPSA) for tomato 76R (+AM) and *rmc* (-AM) genotypes over DAP 13-42 grown using three P sources: OM-P, OM/IN-P and IN-P, grouped horizontally by N rates (LN and HN) and P rates (LP and HP). The width of the ribbons is equal to the least significant differences within a DAP. The predictions are not significantly different (*p* > 0.05) where the ribbons overlap.

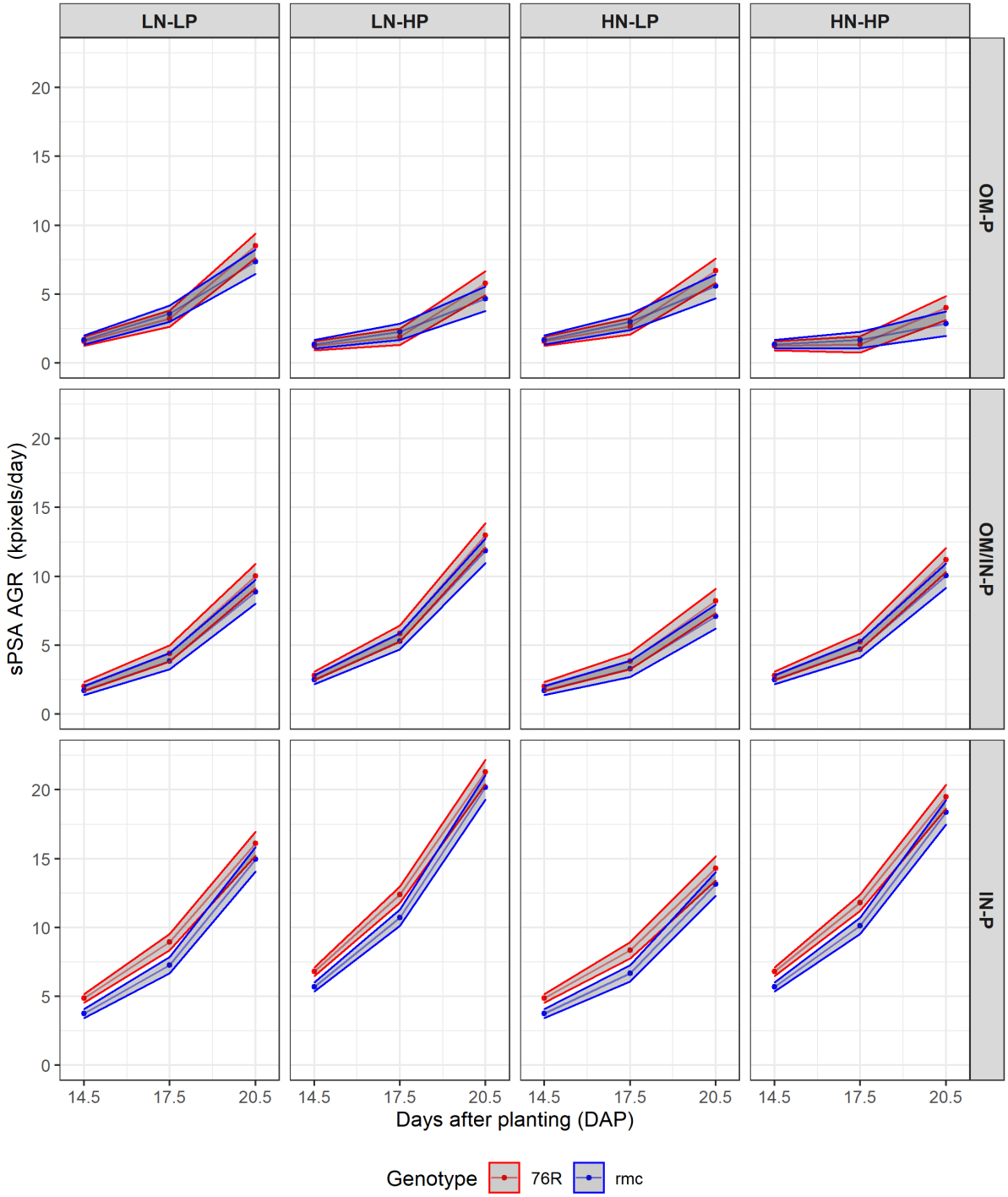


**Fig. 5.** Phenotypic predictions for the smoothed absolute growth rates (sPSA AGR, plotted at DAP interval midpoints) for tomato 76R (+AM) and *rmc* (-AM) genotypes over DAP 13-42 grown using three P sources: OM-P, OM/IN-P and IN-P; and grouped horizontally by N rates (LN and HN) and P rates (LP and HP). The width of the ribbons is equal to the least significant differences within a DAP. The predictions are not significantly different (*$p$* > 0.05) where the ribbons overlap.

### *3.2. Phosphorus source and rate had different effects on soil P availability, AM colonisation and plant biomass between low and high N rate*

At the time of planting, the soils with the HP application rate had higher plant-available P compared to LP rate, and the plant-available P was lowest in the OM-P source and intermediate in the OM/IN

source, regardless of P rate (Table S2). The pattern of shoot P content was similar to that of plant-available soil P, although the differences in shoot P contents among three P sources separated more in the HP than the LP rate. (Fig. S3).

There was a three-way interaction of P source, P rate and N rate on shoot fresh weight (SFW) at the time of harvest (Table S1), and the pattern of SFW was mirrored with the final day of HTP data (Fig. 7, Fig. S1). Values of SFW were similar among different P sources at the LP rate, whereas SFW at the HP rate in the IN-P source was lower or similar compared to the OM-P sources in the LN rate and HN rate, respectively. Root fresh weight (RFW) at the time of harvest was affected by the interaction of P source and rate, in which RFW was greatest in IN-P, intermediate in OM/IN-P and lowest in OM-P treatments, regardless of N rate.

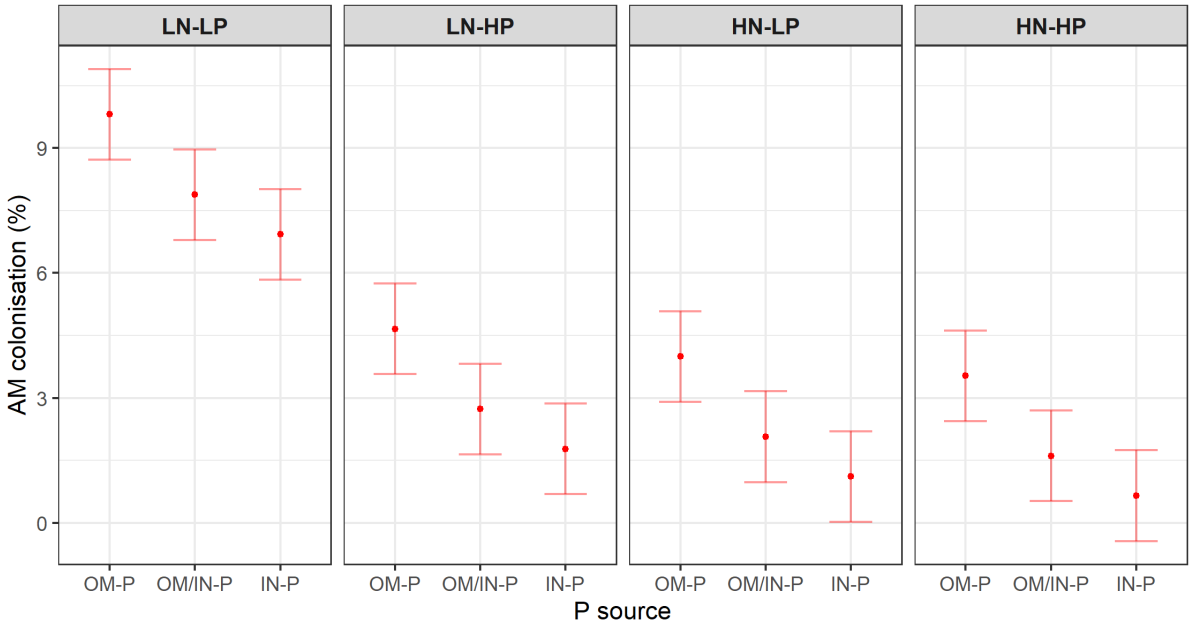


**Fig. 6.** Phenotypic prediction of percent AM colonisation of tomato 76R (+AM) genotype at the destructive harvest grown using three P sources: OM-P, OM/IN-P and IN-P; and grouped by N rates (LN and HN) and P rates (LP and HP). Error bars correspond to half of the least significant difference [±half-LSD (5%)]. Error bars that overlap indicate that the predictions are not significantly different.

The roots of the +AM plants had low colonisation by AM fungi across all treatments; the greatest mean value was 9.8 % root length colonised in the LN/LP treatment of the OM-P source and AM colonisation of roots was reduced when either N or P was applied at high rates, irrespective of P source. (Fig. 6). In addition, of the same P source and rate treatments, the +AM plants had similar shoot biomass, shoot P contents as -AM plants, regardless of N rate, but +AM plants had higher root biomass, compared to -AM plants (Fig. 7, Table S1, Fig. S3).

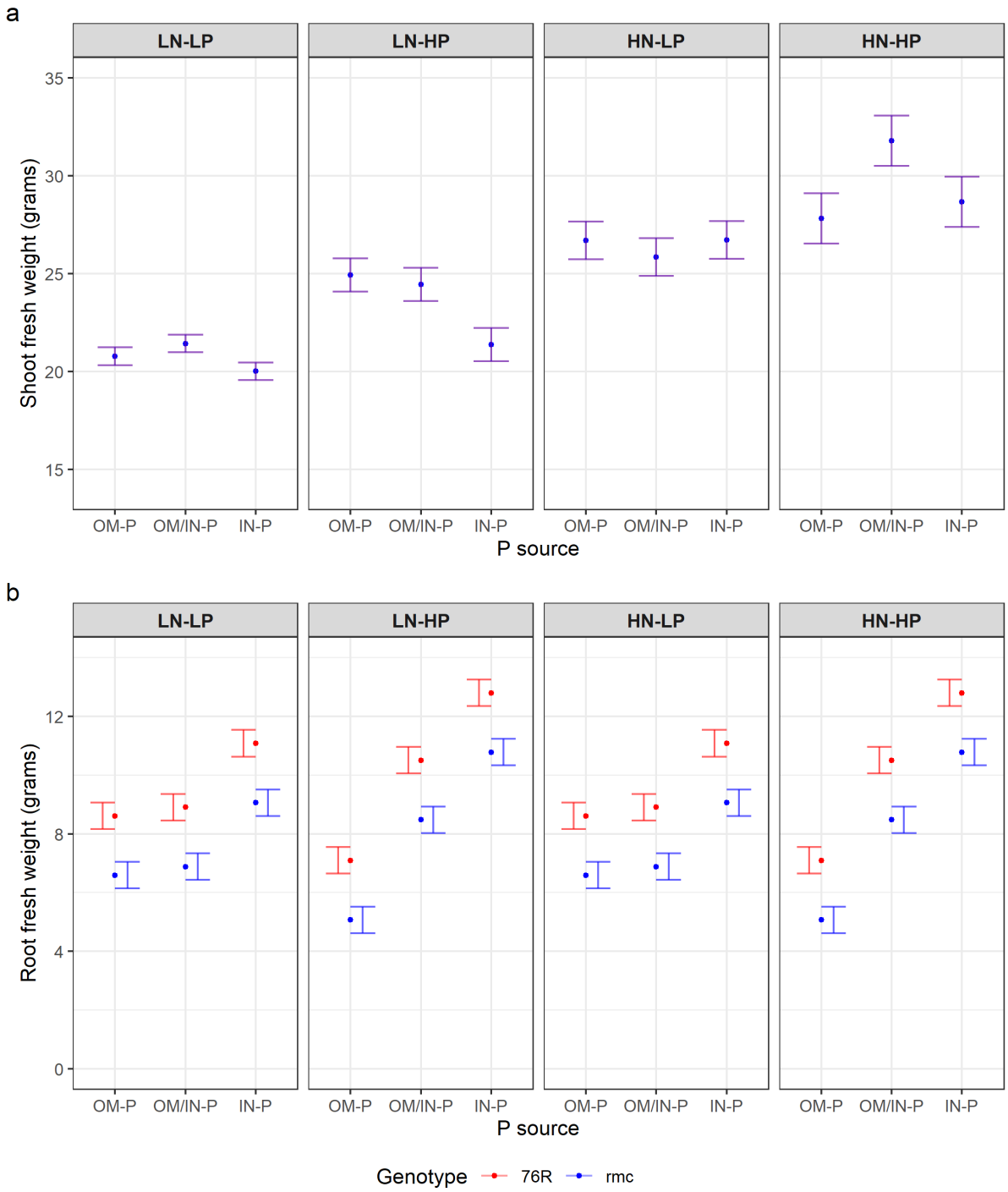


**Fig. 7.** Phenotypic prediction of shoot fresh weight (a) and root fresh weight (b) at the destructive harvest for tomato 76R (+AM) and *rmc* (-AM) genotypes grown using three P sources (OM-P, OM/IN-P and IN-P) and grouped by N rates (LN and HN) and P rates (LP and HP). Error bars correspond to half of the least significant difference [±half-LSD (5%)]. Error bars that overlap indicate that the predictions are not significantly different. N.B. Predicted means and error bars of 76R and *rmc* are overlapped in (a).

## 4. Discussion

Whereas early shoot growth responses to the soil nutrient treatments (P source, P rate and N rate) were stable, the growth responses changed in the later stages. By the time of the destructive

harvest, there were no differences in shoot biomass of the +AM and -AM plants in any of the nutrient addition treatments, even though there were clear differences in shoot growth between +AM and -AM plants at the early time points. These data highlight the complex and temporally dynamic responses of plants to soil P availability (source and rate), N rate, and AM colonisation. In doing so, they also highlight the power and value of high throughput phenotyping in elucidating plant growth and mycorrhizal responses over time.

### *4.1. Shoot growth over time reflected the dynamic nature of soil P availability*

The nature of inorganic and organic sources of P had a large impact on the amount of plant-available P in the soil. Here, the organic P source provided up to 50% of plant-available P, compared to the inorganic P source, despite their being applied to the soil in equal (total P) amounts. This agrees with previous research where organic P sources supplied less rapidly-available P to plants compared to an inorganic P source (Malik *et al.*, 2012). This was not unexpected given that only 37 % of total P in the chicken litter used here is present in a plant-available form (Mackay *et al.*, 2017), and the release of available P to the soil from organic materials is gradual due to microbial P turnover (Achat *et al.*, 2010; Dey *et al.*, 2019). In addition, there was a subsequent impact on plant P uptake, where plants grown with the organic P source did not perform as well as those supplied with the inorganic P source, consistent with previous research (Mackay *et al.*, 2017). It is important to provide nutrients to plants at a time corresponding to plant demand; limitation of nutrient supply at critial developmental stages may reduce biomass accumulation and plant fitness (Martinez *et al.*, 2005; Yan *et al.*, 2019). Thus, the lower shoot growth in P-rich organic source compared to the inorganic P sources was likely a consequence of lower available P supplied to the plants at key developomnetal timepoints.

On the other hand, the combined use of inorganic and organic sources of P resulted in the same, or better, shoot growth (both over time and at destructive harvest) as compared to the organic P source alone; this is likely due to inorganic P source supplying P early, and organic P source supplying P to the plants later. In addition, the combined use of inorganic and organic sources of P possibly reduced the C:P ratio of the amendment, which in turn may have enhanced microbial P mineralisation and plant P uptake (Zhang *et al.*, 2014). However, shoot growth rates of plants given the P-rich organic source were similar to those in the earlier growing stages of the plants given the inorganic P source, indicating shoot growth was stimulated once more available P was mineralised from the organic material. This was supported by the later, but greater, value of sPSA $AGR_{max}$ in the organic P source compared to the inorganic P source. Given this, there are likely important impacts of P source on crop production and the time taken for plants to reach maturity (phenology).

In the later stages of the experiment, plants in the HN treatment group had improved shoot growth compared to the plants in LN treatment (Fig. S1), and the N level interacted with P source from 36 DAP (Table S1). Nitrogen demand is known to change during plant development (Garnett *et al.*, 2013), which may explain why shoot growth in the LN treatment was lower from 36 DAP onwards, compared to shoot growth in the HN treatment. Plants grown with low N and organic P treatment, had increased shoot growth compared to those in the IN-P treatment group, in the late stages of growth, suggesting that the organic material may have had residual N present.

### *4.2. Arbuscular mycorrhizal effects on shoot growth moved from positive to neutral over time*

Final biomass at harvest suggest that AM colonisation had little impact on biomass production and P nutrition of tomato shoots. Conversely, the HTP data suggests that growth over time was indeed affected by AM colonisation, with AM plants that grew faster than non-AM plants in the earlier stages of crop growth. Arbuscular mycorrhizal fungi are able to colonise roots within three days (Resendes *et al.*, 2008) and are able to expand to half of the root system within 14 days (Pérez-de-Luque *et al.*, 2017). In the current work there is evidence that AM colonisation not only has the capacity to form rapidly in tomato roots, but can also confer an advantage to shoot biomass early on. For example, there was a positive shoot growth response in the AM plants as early as 13 days after they were transplanted in the N- and P-limited soil. The positive shoot growth responses aligned with greater AM colonisation in the roots from those plants. However, the positive effect on shoot growth moved to a neutral effect by 22 DAP, and by the end of the experiment, there were no differences in shoot biomass based on AM colonisation. Generally, the 76R tomato genotype displays low biomass responsiveness to AM colonisation, based on studies that only measured biomass at harvest (Watts-Williams and Cavagnaro, 2015); we have shown here that positive responses to AM colonisation were recorded during early stages of plant growth, but disappeared by harvest, thus highlighting the temporal nature of AM responsiveness. In addition, the early positive shoot growth effect could contribute to improved tomato yields in AM plants, even where there were no clear differences in shoot growth at the time of harvest (Bowles *et al.*, 2016). Thus, further research is required to understand potential controls over AM growth and yield effects, over the entire plant growth cycle.

While the effect of AM colonisation on shoot growth at the time of harvest was neutral, its effect on root growth was positive, which may be explained by a positive AM effect on root length (Saia *et al.*, 2020) and root branching (Ramírez-Flores *et al.*, 2019). This difference in effects of AM fungi on above- vs. below-ground biomass has also been seen in previous research (Tran *et al.*, 2019), and hence it would be useful to use non-destructive root phenotyping to measure root

growth in the future, to fully understand the temporal effects of AM fungi on a range of plant species.

## Conclusions

We used a high-throughput phenotyping system to investigate tomato shoot growth over time, in plants fertilised with three different P sources, and with or without AM associations. The inorganic P source alone led to rapid shoot growth over time compared to the P-rich organic source alone, or the combination of the two sources, which was likely due to the ready P availability of the inorganic P source. However, while maximum shoot growth was delayed in plants supplied with the organic P source, maximum growth rates were higher, indicating shoot growth was stimulated once additional available P had been mineralised from the organic material. Our data highlight the benefit of AM associations in early shoot growth of tomato plants, likely due to increased nutrient access while the root system is small. Taken together, the results suggest that the combined use of P-rich organic materials and inorganic P sources can be used to close the growth gap and time gap effects between organic and inorganic P sources, and that maintaining high levels of AM colonisation could be a useful way to benefit tomato crops.

## Supplementary data

Table S1. The p-values for the corresponding Wald F-statistics of $2^4$-1=15 treatment main effects and interactions, followed by the overall chosen model.

Table S2. Characteristics of the soil at the time of planting after incorporating three P sources: OM-P, OM/IN-P and IN-P.

Fig. S1. Phenotypic predictions for the smoothed projected shoot area (sPSA) plotted separately from DAP 13 to DAP 42 DAP for tomato 76R (+AM) and rmc (-AM) genotypes grown using three P sources.

Fig. S2. Phenotypic predictions for the smoothed absolute growth rates (sPSA AGR) plotted separately for the defined time periods for tomato 76R (+AM) and rmc (-AM) genotypes grown using three P sources.

Fig. S3. Phenotypic prediction for shoot P content at the destructive harvest for tomato 76R (+AM) and rmc (-AM) genotypes grown using three P sources.

Fig. S4. Phenotypic prediction of shoot dry weight (a) and root dry weight (b) at the destructive harvest for tomato 76R (+AM) and rmc (-AM) genotypes grown using three P sources.

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## Author Contributions

HTTN, SJWW, BB and TRC conceptualized the study and designed experiments. HTTN performed experiments. HTTN, NJ and CB analysed data. HTTN wrote the manuscript. All the authors have read and edited the final manuscript.

## Data availability statement

Data sharing is not applicable to this article as all created data is already contained within this article or in the supplementary material.

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## Supplementary

**Table S1.** The $p$-values for the corresponding Wald F-statistics of $2^4 - 1 = 15$ treatment main effects and interactions, followed by the overall chosen model. Statistically significant terms ($p \leq 0.05$) are flagged with an asterisk (*). Wherever an interaction term is significant, the corresponding lower-level $p$-values are marked as not applicable (na).
G: tomato genotype, N: nitrogen application rate, P: phosphorus application rate and S: phosphorus source

| Trait | G:N:P:S | G:N:P | G:N:S | G:P:S | N:P:S | G:N | G:P | G:S | N:P | N:S | P:S | G | N | P | S | Chosen model |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| sPSA 13 | 0.708 | * 0.010 | 0.197 | 0.327 | 0.496 | na | na | 0.712 | na | 0.607 | * 0.019 | na | na | na | na | P:S + G:N:P |
| sPSA 16 | 0.809 | * 0.015 | 0.210 | 0.519 | 0.463 | na | na | 0.170 | na | 0.697 | * <0.001 | na | na | na | na | P:S+ G:N:P |
| sPSA 19 | 0.757 | 0.056 | 0.217 | 0.641 | 0.529 | 0.158 | 0.775 | 0.051 | 0.204 | 0.727 | * <0.001 | * <0.001 | 0.380 | na | na | P:S + G |
| sPSA 22 | 0.754 | 0.187 | 0.245 | 0.551 | 0.616 | 0.249 | 0.849 | 0.058 | 0.168 | 0.405 | * <0.001 | * 0.002 | * 0.032 | na | na | P:S + N + G |
| sPSA 25 | 0.785 | 0.338 | 0.301 | 0.480 | 0.735 | 0.484 | 0.779 | 0.109 | 0.221 | 0.277 | * <0.001 | * 0.003 | * 0.003 | na | na | P:S + N + G |
| sPSA 30 | 0.759 | 0.459 | 0.470 | 0.596 | 0.926 | 0.973 | 0.750 | 0.338 | 0.588 | 0.708 | * <0.001 | * 0.002 | * 0.004 | na | na | P:S + N + G |
| sPSA 36 | 0.783 | 0.461 | 0.718 | 0.810 | 0.150 | 0.609 | 0.901 | 0.890 | 0.590 | * 0.008 | * <0.001 | * 0.012 | na | na | na | P:S + N:T + G |
| sPSA 42 | 0.993 | 0.968 | 0.520 | 0.827 | * 0.004 | 0.364 | 0.524 | 0.771 | na | na | na | 0.114 | na | na | na | N:P:S |
| sPSA AGR 13-16 | 0.569 | 0.071 | 0.369 | 0.758 | 0.531 | 0.191 | 0.750 | * 0.008 | 0.109 | 0.861 | * <0.001 | na | 0.451 | na | na | P:S + G:S |
| sPSA AGR 16-19 | 0.620 | 0.352 | 0.348 | 0.529 | 0.606 | 0.208 | 0.928 | * 0.022 | 0.094 | 0.369 | * <0.001 | na | * 0.050 | na | na | P:S + G:S + N |
| sPSA AGR 19-22 | 0.789 | 0.641 | 0.363 | 0.382 | 0.615 | 0.467 | 0.955 | 0.104 | 0.170 | 0.124 | * <0.001 | * 0.029 | * <0.001 | na | na | P:S + N + G |
| sPSA AGR 22-25 | 0.859 | 0.759 | 0.470 | 0.406 | 0.745 | 0.944 | 0.692 | 0.272 | 0.381 | 0.173 | * <0.001 | * 0.015 | * <0.001 | na | na | P:S + N + G |
| sPSA AGR 25-30 | 0.746 | 0.815 | 0.873 | 0.881 | 0.341 | 0.261 | 0.766 | 0.832 | 0.537 | 0.770 | * <0.001 | * 0.011 | * 0.033 | na | na | P:S + N + G |
| sPSA AGR 30-36 | 0.804 | 0.717 | 0.972 | 0.831 | * <0.001 | 0.335 | 0.471 | 0.297 | na | na | na | 0.671 | na | na | na | N:P:S |
| sPSA AGR 36-42 | 0.708 | 0.393 | 0.620 | 0.954 | * <0.001 | 0.145 | 0.522 | 0.439 | na | na | na | 0.216 | na | na | na | N:P:S |
| Shoot fresh weight | 0.336 | 0.938 | 0.409 | 0.825 | * 0.008 | 0.229 | 0.493 | 0.682 | na | na | na | 0.383 | na | na | na | N:P:S |
| sPSA AGR Max | 0.972 | 0.738 | 0.361 | 0.721 | * <0.001 | 0.395 | 0.953 | 0.840 | na | na | na | 0.848 | na | na | na | N:P:S |
| sPSA AGR Max DAP | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | (P:S + G)[LN] + (P)[HN] |
| Shoot dry weight | 0.736 | 0.803 | 0.423 | 0.768 | 0.471 | 0.773 | 0.587 | 0.832 | 0.968 | 0.716 | * <0.001 | * 0.013 | * <0.001 | na | na | P:S + N + G |
| Root fresh weight | 0.973 | 0.378 | 0.912 | 0.192 | 0.192 | 0.110 | 0.645 | 0.315 | 0.428 | 0.056 | * <0.001 | * <0.001 | 0.229 | na | na | P:S + G |
| Root dry weight | 0.830 | 0.376 | 0.814 | 0.563 | 0.234 | 0.675 | 0.921 | 0.149 | * 0.014 | * 0.007 | * <0.001 | * <0.001 | na | na | na | P:S + N:S + N:P + G |
| AM colonisation | na | na | na | na | 0.096 | na | na | na | * 0.012 | 0.957 | 0.696 | na | na | na | * 0.027 | S + N:P |
| Shoot P content | 0.526 | 0.291 | 0.213 | 0.401 | 0.128 | 0.822 | 0.913 | 0.484 | * 0.034 | 0.559 | * <0.001 | 0.741 | na | na | na | P:S + N:P |

**Table S2.** Characteristics of the soil at the time of planting after incorporating three P sources: OM-P, OM/IN-P and IN-P. Values are mean ± SE, *n* = 10. Within column, means followed by different letters are significantly different at the P < 0.05 level.

| P rate | P source | Plant-available P (mg $kg^{-1}$) Day 0 | pH | EC (µS/cm) | Available N (mg $kg^{-1}$) |
|---|---|---|---|---|---|
| LP | IN-P | 11.86±1.68(b) | 6.49±0.03(ab) | 111.3±6.59(b) | 26.89±0.65(c) |
| | OM/IN-P | 8.52±1.63(bc) | 6.54±0.09(ab) | 108.5±4.90(b) | 28.85±0.85(c) |
| | OM-P | 2.75±1.32(c) | 6.48±0.14(b) | 110.3±4.86(b) | 30.51±0.86(c) |
| HP | IN-P | 23.01±1.98(a) | 6.24±0.04(c) | 108.9±3.38(b) | 27.12±1.00(c) |
| | OM/IN-P | 14.73±1.36(b) | 6.47±0.03(b) | 120.1±3.01(ab) | 38.50±2.53(b) |
| | OM-P | 12.98±3.34(b) | 6.71±0.03(a) | 128.3±5.08(a) | 54.88±5.01(a) |

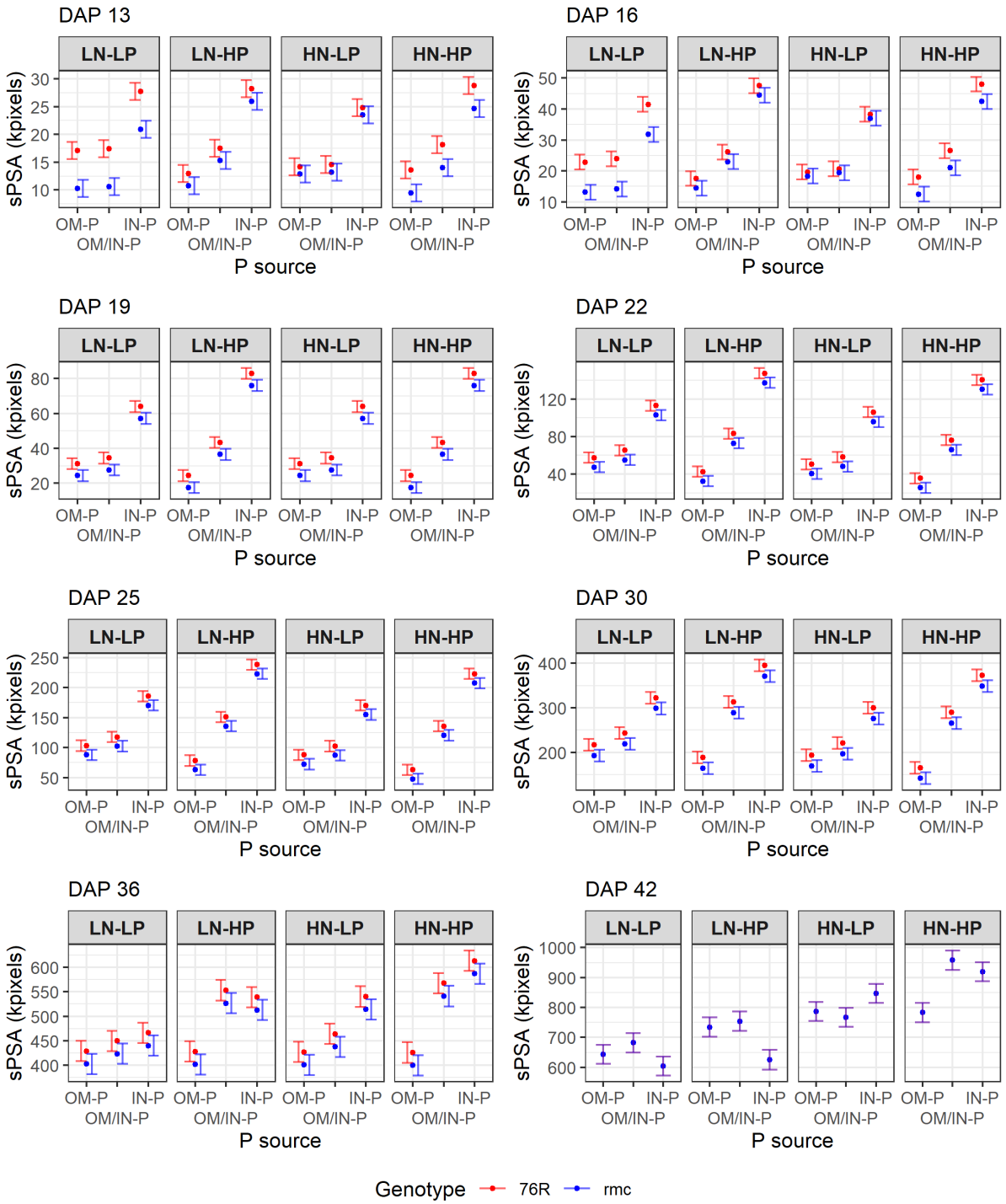


**Fig. S1.** Phenotypic predictions for the smoothed projected shoot area (sPSA) plotted separately from DAP 13 to DAP 42 DAP for tomato 76R (+AM) and rmc (-AM) genotypes grown using three P sources: OM-P, OM/IN-P and IN-P, grouped horizontally by N rates (LN and HN) and P rates (LP and HP). Error bars correspond to half of the least significant difference [±half-LSD (5%)]. Error bars that overlap indicate that the predictions are not significantly different.

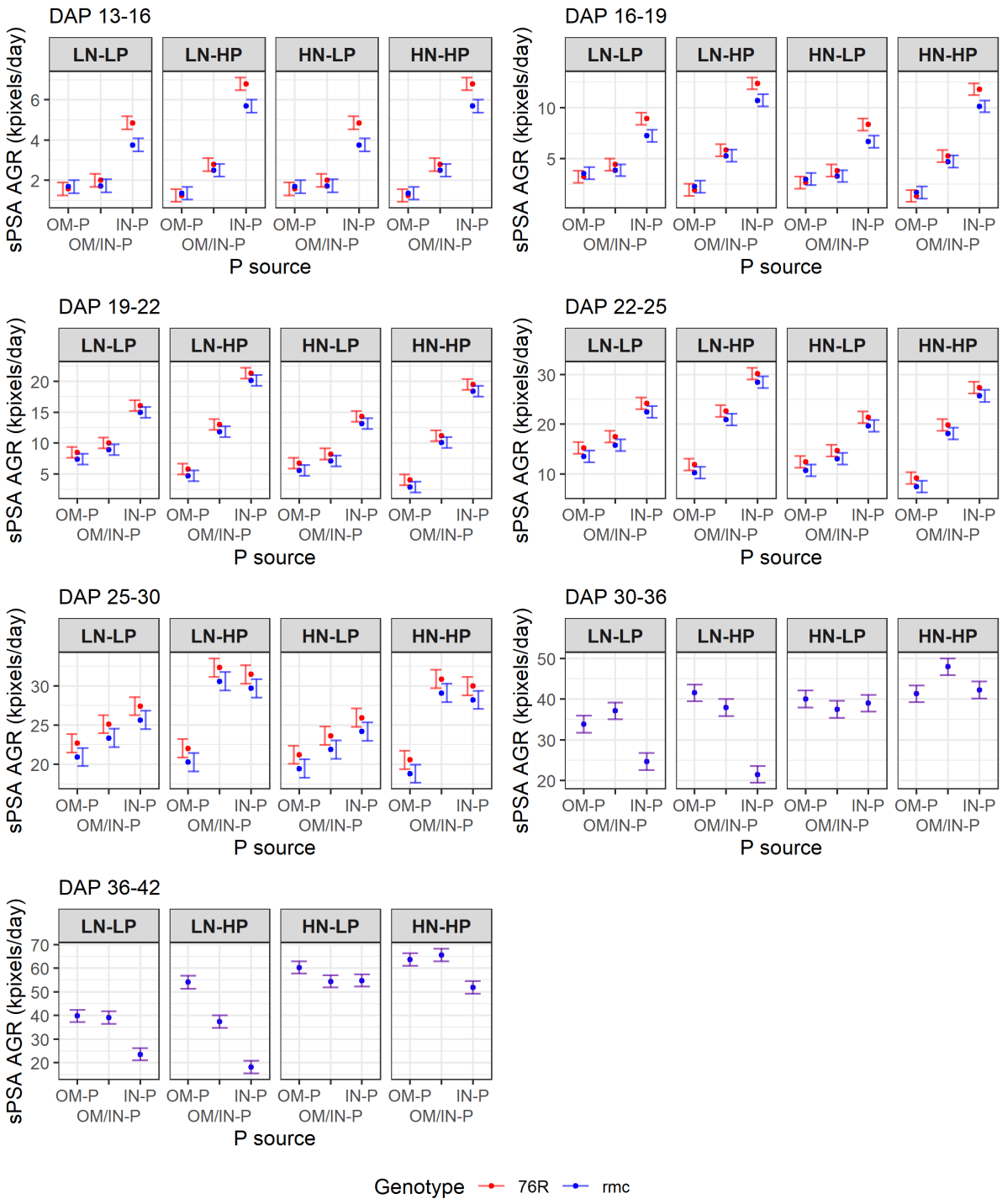


**Fig. S2.** Phenotypic predictions for the smoothed absolute growth rates (sPSA AGR) plotted separately for the defined time periods for tomato 76R (+AM) and *rmc* (-AM) genotypes grown using three P sources: OM-P, OM/IN-P and IN-P, grouped horizontally by N rates (LN and HN) and P rates (LP and HP). Error bars correspond to half of the least significant difference [±half-LSD (5%)]. Error bars that overlap indicate that the predictions are not significantly different.

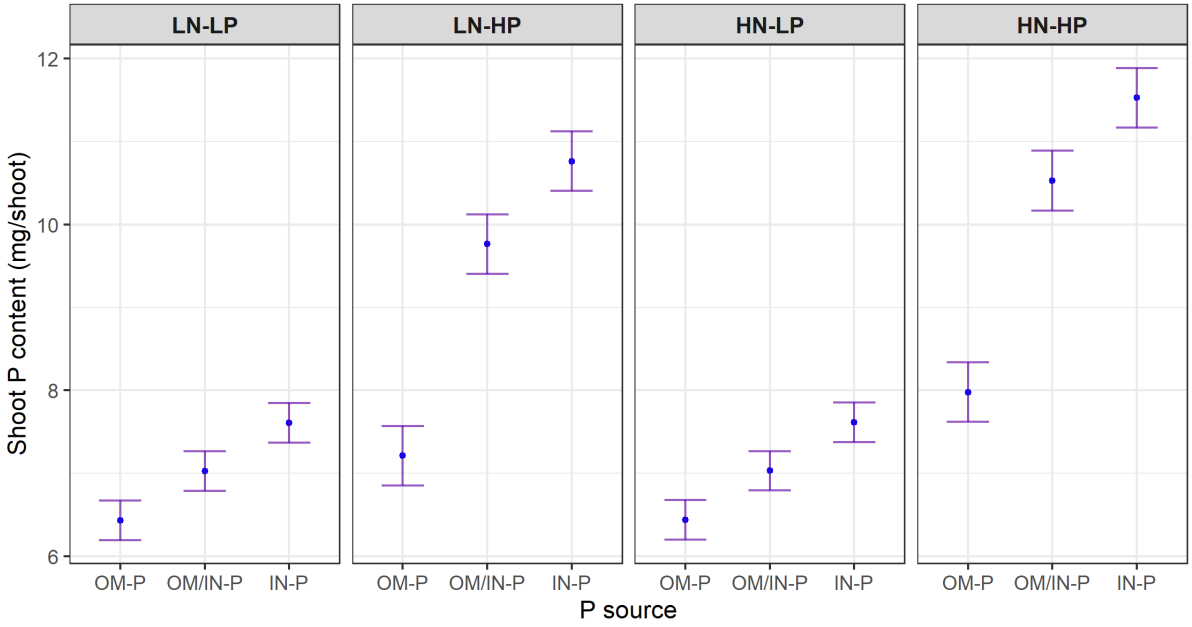


**Fig. S3.** Phenotypic prediction for shoot P content at the destructive harvest for tomato 76R (+AM) and *rmc* (-AM) genotypes grown using three P sources: OM-P, OM/IN-P and IN-P; and grouped by N rates (LN and HN) and P rates (LP and HP). Error bars correspond to half of the least significant difference [±half-LSD (5%)]. Error bars that overlap indicate that the predictions are not significantly different. N.B. Predicted means and error bars of 76R and *rmc* fully overlapped in (a).

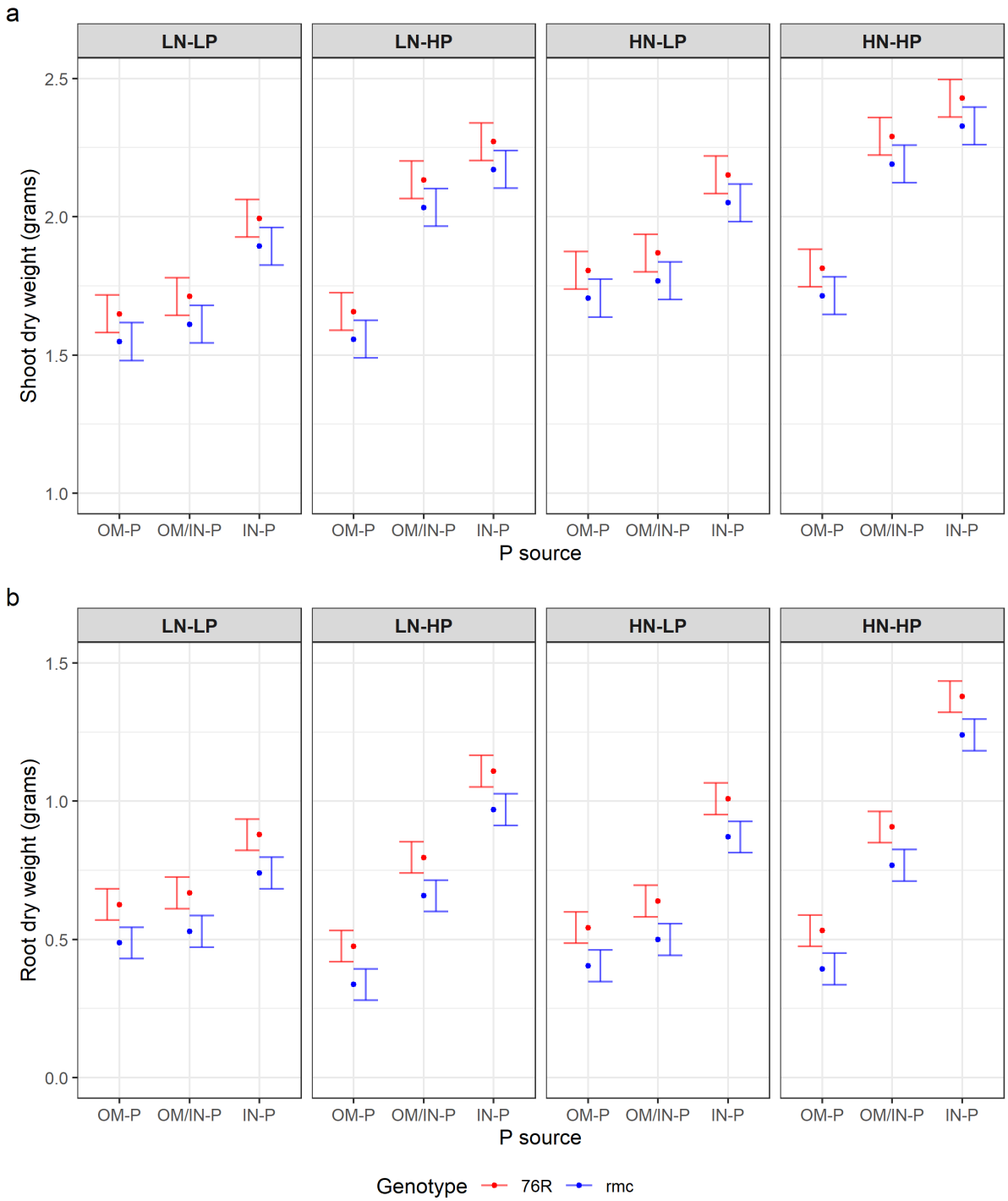


**Fig. S4.** Phenotypic prediction of shoot dry weight (a) and root dry weight (b) at the destructive harvest for tomato 76R (+AM) and *rmc* (-AM) genotypes grown using three P sources: OM-P, OM/IN-P and IN-P, and grouped by N rates (LN and HN) and P rates (LP and HP). Error bars correspond to half of the least significant difference [±half-LSD (5%)]. Error bars that overlap indicate that the predictions are not significantly different.

# CHAPTER 5: FORMULATION AND EVALUATION OF CHICKEN LITTER/MAP ORGANOMINERAL FERTILISERS.

# Statement of Authorship

| | |
|---|---|
| Title of Paper | Formulation and evaluation of chicken litter/MAP organomineral fertilisers. |
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**Formulation and evaluation of chicken litter/MAP organomineral fertilisers.**

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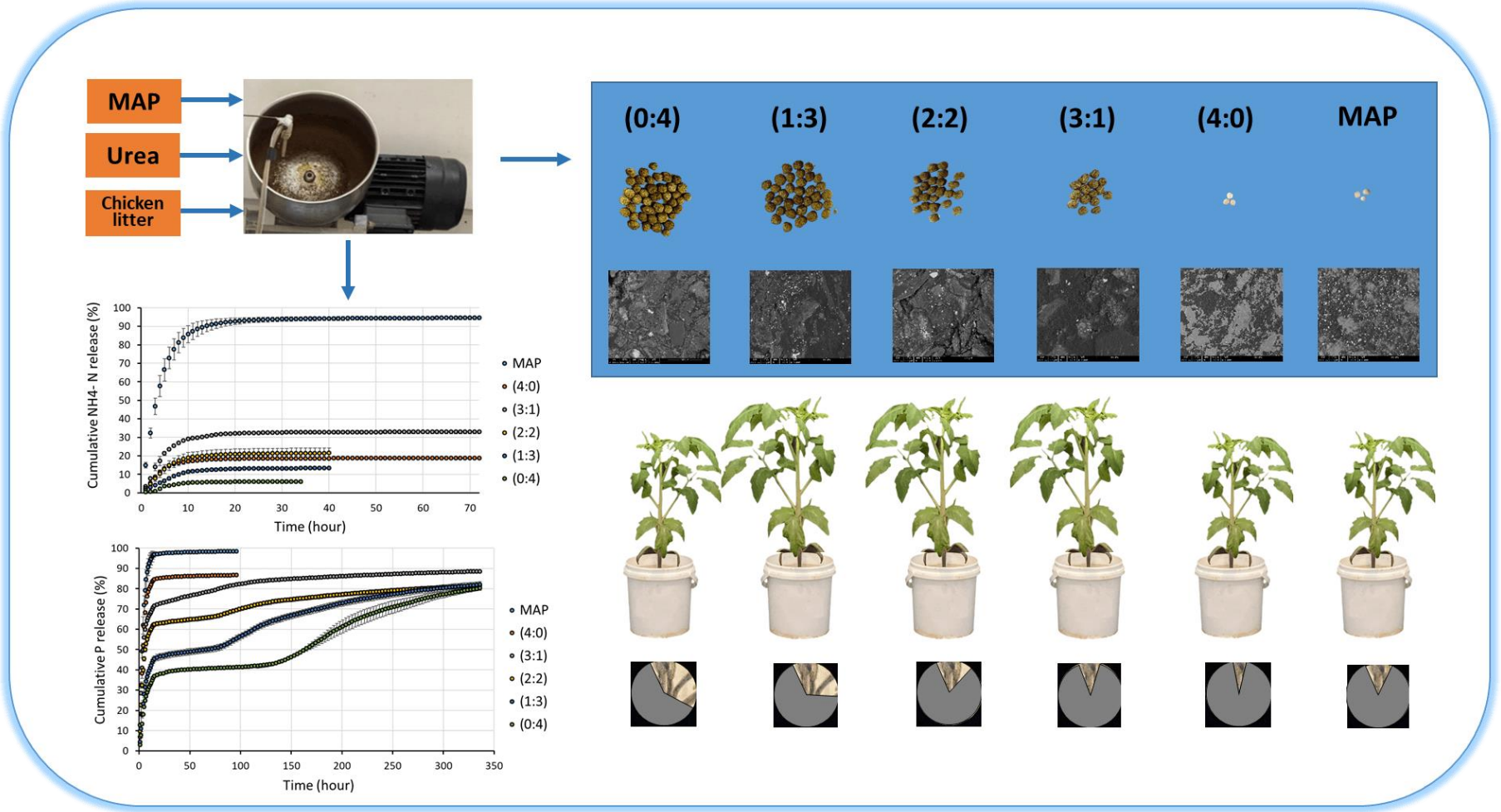
## Abstract

Managing sustainable agricultural development requires the efficient use of nutrient resources, in which recovering nutrients for crop production from animal manures plays a key role. Animal manures usually have low nutrient contents, often not ideal for balanced crop nutrition, and the physical form of the manure often makes transport and spreading difficult. Here, we formulated organomineral fertilisers to produce granular products with a sizing suitable for transport and spreading. We also determined effects on arbuscular mycorrhizal colonisation of plants as P-rich organic fertilisers were previously found to suppress colonisation. Formulated fertilisers were produced by granulating chicken litter powder with inorganic fertiliser powders (MAP) in different ratios, namely 0:4, 1:3, 2:2, 3:1, 4:0. The N:P ratios were balanced in the products by co-granulating urea into the formulations and a MAP only control (no additional urea) was also included. Physical and chemical characteristics of formulations were measured. In addition, the kinetics of N and P release from formulated fertilisers were determined before testing plant growth, nutrition and arbuscular mycorrhizal responses. After about 12 hours, no more $NH_4^+$-N was dissolute from all formulations, however, the maximum cumulative $NH_4^+$-N release was lower in the formulations that were high in chicken litter. Whereas, the maximum cumulative P release was about 80% in all formulations and the time to obtain maximum P dissolution was extended to 19 days in the formulation that contained only chicken litter. Consequently, the kinetics of $NH_4^+$-N and P release affected plant growth, nutrition and mycorrhizas such that organomineral formulations increased shoot growth by 15- 28% compared to the chicken litter only, MAP/urea only and MAP only formulations. Organomineral formulations also had good AM colonisation of roots. The present study showed that better plant growth did not depend on immediately available nutrients, it was more likely related to timing supply of nutrients to match plant demand. The combination of chicken litter with MAP sustained nutrient supply and improved plant growth and therefore the use of organomineral fertilisers in agriculture should be encouraged. Taken together, organomineral formulations are alternative P fertilisers that provide a potential solution for recycling agricultural wastes while contributing to crop nutrition.

**Keywords:** Agricultural wastes; Arbuscular mycorrhizal fungi; Monoammonium phosphate; Phosphorus; Recycling; Tomato

## Graphical abstract



## 1. Introduction

The use of fertilisers to maximise crop yields has revolutionised agriculture. Fertilisers are applied to the soil to bolster and/or replenish essential nutrient elements (Hopkins and Hansen, 2019). In many cases, nutrients are applied in excess of actual plant demand (Nash *et al.*, 2019; Smil, 2000). This is in part because most P in fertilisers is released quickly after application (Lombi *et al.*, 2005; Williams, 1969) and so more is added to meet the ongoing demand of a plant over its life cycle. Moreover, P binds readily to the surface of soil particles (Asomaning, 2020), and can form relatively insoluble precipitates with soil cations (Hawkesford *et al.*, 2012), which render it largely unavailable to plants. It is for these reasons that plants only take up an average of 15- 30% total P fertiliser applied in the year of application (Dhillon *et al.*, 2017; White, 2009). However, P not taken up by plants, for example due to precipitation by soil cations such as Fe, Al and Ca, can increase the total P pool in the soil (Lopez-Arredondo *et al.*, 2014; McLaughlin *et al.*, 2011). This residual P is then at risk of loss via leaching and/or surface runoff, which can lead to algal blooms, hypoxia and/or eutrophication when it reaches water bodies (Daloğlu *et al.*, 2012; Fink *et al.*, 2016; Garnache *et al.*, 2016; Khan *et al.*, 2018; Scavia *et al.*, 2014).

In addition to the economic and environmental implications of inefficient P use in agriculture, much of the world's inorganic P fertilizers are produced using a finite resource - phosphate rock (Lu and Tian, 2017). While there is ongoing discussion regarding the timeline of mineral P reserve depletion (Koppelaar and Weikard, 2013), there is nevertheless the need to use finite resources efficiently. Moreover, the production of mineral P fertilisers involves the process of acidulation with sulphuric acid. As we transition towards renewable (i.e. non-fossil fuel) energy sources, S (a by-product of fossil fuel production) will also become increasing scarce (Zimmerman, 1977). Taken together, there is a clear need to use P fertiliser as efficiently as possible, and to identify alternative sources of P with which to fertilise crops.

The use of P-rich waste materials, including technology to substitute raw materials with residual biomass (Chojnacka *et al.*, 2020), as a source of P for agriculture, has been the subject of much research. For example, organic wastes containing N and P, as well as organic C, are used in many agricultural systems as fertilisers (Grigatti *et al.*, 2019; Mackay *et al.*, 2017b). In addition to recycling valuable nutrients in agricultural wastes, the use of such materials as fertilisers can be less expensive than the costs associated with waste disposal (Antille *et al.*, 2013). One drawback of using P-rich waste materials, such as chicken litter as a source of nutrient in agriculture, is that their need for nutrients to be mineralised before they can be taken up by plants (Quilty and Cattle, 2011). As a result, nutrients may not be present in a plant-available form early in the crop growth cycle when demand is high, or in the right stoichiometry for balanced crop nutrition (Redding *et al.*, 2016). For chicken litter, and indeed other soil organic amendments, to be used to maximum effect as a

fertiliser, there is a need to develop processes for converting P-rich waste materials into efficient phosphatic fertilisers (Ashworth *et al.*, 2020; Ndambi *et al.*, 2019). One such approach is the production of organomineral fertilisers (Chojnacka *et al.*, 2020).

Organomineral fertilisers - the combined use of chicken litter with inorganic fertilisers - have been found to have similar or better effects on plant growth, compared to the use of inorganic fertilisers alone (Crusciol *et al.*, 2020; Frazão *et al.*, 2019). This is because the inorganic P source provides an immediate supply of plant-available P, which is important during the initial growth stage where plant root systems have not been fully developed, and the organic P sources gradually provides P (via mineralisation) to support later stages of plant growth (Akhtar *et al.*, 2011; Grant *et al.*, 2001; Moe *et al.*, 2017; Zhang *et al.*, 2017). Organomineral fertilisers produced from chicken litters and soluble inorganic P sources show slow P release characteristics and perform differently in soils compared to inorganic or organic formulations (Mazeika *et al.*, 2016; Rodrigo Sakurada *et al.*, 2019). The efficient use of organomineral fertilisers will be maximised where P release kinetics are synchronised with plant demand. It is also important that organomineral fertilisers are formulated (e.g. as granules) such that they can be delivered using existing farm equipment (Nascimento *et al.*, 2021).

In addition to the formulation and efficient use of P fertilisers, be they organomineral fertilisers or not, there are other opportunities to maximise plant P acquisition. For example, it is well established that the formation of arbuscular mycorrhizas (AM) can help to improve plant P acquisition (Rillig *et al.*, 2016). Arbuscular mycorrhizal fungi (AMF) transport inorganic P (Pi) into plant roots via the mycorrhizal pathway, a unique Pi transport channel from the soil into root cells that can contribute up to 100% of overall plant P uptake (Schnepf *et al.*, 2008; Smith, 2003). The hyphae of AMF are able to scavenge for P beyond root depletion zones as they can extend their growth up to 30 mm from the root surface (Mai *et al.*, 2019), thereby gaining access to P that may be otherwise unavailable to roots. However, AM fungi are obligate symbionts, meaning that plants need to exchange C and lipid sources for the gain of P or other nutrients from AM fungi (Smith and Read, 2008). Consequently, depending on soil conditions and plant species, AM colonisation can have diverse effects on plant growth, ranging from positive to negative (Treseder, 2013). The formation of AM tends to be reduced in soils with high available P, including agricultural systems where inorganic P inputs are high (Gosling *et al.*, 2013; Kobae *et al.*, 2016). It has been suggested that the slow release of P from organic amendments may help reduce the negative impacts on the formation of AM (Mackay *et al.*, 2017a).

Taken together, there is a need to produce organomineral fertilisers that are fit for purpose; that is, they can be delivered using existing equipment, release P in a predictable manner, and they improve plant growth and nutrition, while not affecting AM. Therefore, here we present results of a study in which we sought to:

- Formulate organomineral fertilisers comprised of co-granulated chicken litter and MAP;
- Characterise the physical and chemical properties of the formulated fertilisers, including N and P release kinetics and elemental mapping of the fertiliser granules; and
- Quantify the effects of the organomineral fertilisers on plant growth, nutrition and the formation and functioning of AM.

We hypothesize that organomineral formulations will have dual release characteristics that sustain available P to plants over the growing period and support growth of both plants and AMF.

## 2. Materials and Methods

### *2.1. Granular fertiliser production*

Powders of commercial MAP fertiliser (<250 µm), urea powder (<250 µm) and chicken litter (ground to <290 µm) were used to make organomineral fertilisers (Table S1). The organo-mineral fertilisers were made by mixing MAP powder and chicken litter powder in different ratios based on their total P provided, namely formulations of 4:0, 3:1, 2:2, 1:3 and 0:4. As the N/P ratio in chicken litter was higher than that of MAP (Table S1), urea powder (<250 µm) was added to the formulations (Table S2) with the exception of the 0:4 treatment to provide a consistent N/P ratio in all formulations. In addition, granules were also formulated using MAP powder, to provide a control inorganic P fertiliser without additional urea. Before granulation, the powder mixture of each formulation was homogenised using a household blender (Auto-IQ, Ninja).

Granular fertilisers were made using a laboratory-scale granulation drum (20 cm wide x 9 cm deep) rotating at an angle 39 degrees and utilizing a binder solution GemCone ™ high dissolved solids air assisted ICP nebuliser fed by a pump. For the MAP and 4:0 formulations, ~10 g of dry powder were placed on the base of the drum and sprayed with deionised (DI) water as a binder solution, then the drum run at a rotational speed 10 rpm. Dry powder was then manually added to growing granules until a targeted size was achieved. For other formulations that contained chicken litter, 10 g of mixed powder were wetted by DI water (~ to approximately 50% w/w) and run in the drum granulator at an adjusted speed to produce ~1 mm diameter seed granules. After that, the drum rotational speed and the nebuliser rate for the binder solution were set at 15 rpm and 0.5 mL per min, respectively. The binder solution was 5% sodium silicate and 0.5% polyethylene glycol (PEG) in DI water (following Chen *et al.* (2019)). Dry powder was added until the targeted size was achieved. After granulation, granules were immediately removed from the drum, sieved to a targeted size range (2.00- 2.36 mm) and dried on trays at room temperature for three days.

### 2.2. Characterisation of organo-mineral fertilisers

Total C and total N concentration were measured using the Dumas method and the concentration of total P, K, Ca, Na, Mg, S, Fe, Al, Mn, Zn, B, Cu, Mo and Co were measured by inductively coupled plasma optical emission spectroscopy (ICP-OES). Granular fertilisers were shaken with 2 M KCl solution (1:250 fertiliser: extractant ratio) for one hour and $NH_4^+$-N concentrations (following Forster (1995)) and NO3--N concentrations (following Miranda *et al.* (2001) were measured. Water-extractable P, acid-extractable P and pH were measured following 16 hours sequential water-acid extraction of individual fertiliser granules (Tiessen and Moir, 1993).

Scanning Electron Microscope (SEM) with Energy Dispersive X-ray analysis (Quanta 450 FEG SEM (FEI Company, Netherlands) was used to examine granule structure and the distribution of nutrient elements in cross sections of the fertiliser granules. Before examination by SEM, representative granules from each formulation were fixed in a transparent epoxy resin, cross sections were cut, and the surface polished. The surfaces were then coated with a thin C layer. In all formulations, the voltage was set at 20 kV and the images were acquired with the Backscattered-Electron (BSE) detector at x100 and x 500 magnifications. Map spectrum of elements was measured for two granules from each formulation. The spectrum values were similar indicating that granules were homogenous. Elemental mapping (C, O, N, P, K, Ca, Na,Mg, Fe, Al, S, Cl, , and Si) was examined for one granule from each formulation.

### 2.3. Kinetic of $NH_4^+$-N and P dissolution from organo-mineral fertilisers

A column perfusion test was used to evaluate the kinetics of $NH_4^+$-N and P release from the organo-mineral fertilisers following Baird *et al.* (2019). Briefly, one gram of each granular formulation was placed in the centre of a polypropylene column (150 mm × 15 mm) and the granules were kept in place by acid-washed glass wool. Deionized water was pumped from the bottom of the column to top at a constant flow rate of 10 mL h–1. The eluate containing dissolved nutrient was collected hourly using an automated fraction collector (SuperFrac, Pharmacia) for up to 336 hours (2 weeks). All formulations were assessed in duplicate. The concentration of $NH_4^+$-N and P in eluates were determined by a colorimetric method following Forster (1995) and by ICP-OES, respectively and the residue remaining in the column after the completed collection time was recovered, analysed and used to establish the total nutrient concentration in the formulation. Eluant concentrations were then expressed as a % of this total concentration.

### 2.4. Plant and arbuscular mycorrhizal response tests

The soil used in this experiment was a sandy loam collected from the Waite Arboretum, South Australia (S34°58’01’’, E138°37’46’’), which was air-dried, sieved (<2 mm), and mixed with fine sand

(1:9 w/w) to reduce plant-available P of the soil (bicarbonate-extractable P = 4.17 mg P $kg^{-1}$) and to facilitate root sampling at the end of the experiment. Prior to the addition of granular fertilisers, 1,330 g soil was weighed for each pot, then thoroughly mixed with 70 g inoculated soil containing propagules of AM isolate *Rhizophagus irregularis* WFVAM10 (DAOM181602). The experimental design was completely randomized with 2x6 factorial treatments and five replicates. The factors corresponded to two plant genotypes (mycorrhizal and non-mycorrhizal) and six fertiliser formulations represented by the proportion of total P in MAP and chicken litter: 0:4 (chicken litter only), 1:3, 2:2, 3:1, 4:0 all with N:P ratios balanced using urea, and a MAP-only control (with no urea). All fertilisers were applied to pots at 78 mg N kg-1 soil and 30 mg P kg-1 soil. Specifically, 3 cm of soil was packed into each pot (1.2 L size, 12 cm depth), then a layer of granular fertilisers was spread on the soil before the remaining soil was packed on top, providing a fertiliser application depth of 7 cm.

Two tomato (*Solanum lycopersicum* L.) genotypes were grown with contrasting abilities to form AM associations with *R. irregularis* WFVAM10. Tomato seedlings were prepared by growing germinated seeds of 76R genotype (able to form association with *R. irregularis* WFVAM10) and *rmc* genotype (unable to form association with *R. irregularis* WFVAM10) in pre-steamed sand substrate (Ngo *et al.*, 2021). After two weeks, one seedling of each tomato genotype was transplanted into experiment pots. All pots were randomly positioned on benches in a glasshouse where conditions were 23.5 °C day and 17.6 °C night with supplemental lighting (1,000 W metal halide lamps) for a 16/8 hour day/night photoperiod. Pots were watered by weight with reverse osmosis (RO) water three times per week to 10 % gravimetric soil moisture content.

On 35 DAP (days after planting), all tomato plants were destructively harvested. Shoots were cut at soil level and weighed, and then dried in an oven at 60 °C for a week, then they were weighed again for dry biomass. The dry shoots were then ground to a fine powder in a puck mill pulveriser and analysed for total P concentration by ICP-OES following digestion in concentrated nitric acid and 36 % hydrogen peroxide (1:4 v/v) (following Wheal *et al.* (2011)). Roots were subsampled and washed free of any attached soil with RO water, then ~200 mg fresh roots were taken for staining AM structures. Firstly, fresh roots were fixed by 50 % ethanol for 24 hours. Then, fixed roots were rinsed with RO and placed in 10 % KOH (W/V) at room temperature to clear the roots, after which the roots were rinsed, stained in 5 % ink in vinegar at 60 °C for 15 min, and de-stained in acidified water for 24 hours, before being stored in 50 % glycerol solution (Vierheilig *et al.*, 1998). Arbuscular mycorrhizal colonisation was estimated on stained root samples at 20 × magnification as percent root length colonised according to the gridline intersect method (Giovannetti and Mosse, 1980). Electrical conductivity (EC) and pH were measured on air-dry soil samples in a 1:5 soil:water suspension. Plant-available (Colwell) P in soil at harvest was measured in an aliquot of a 0.5 M $NaHCO_3$ soil extraction (1:100 soil:extractant) (Murphy and Riley, 1962).

Three plants were eliminated (76R 1:3 formulation, *rmc* 4:0 formulation and *rmc* 3:1 formulation) as the plants had critically low AM colonisation, critically low shoot dry weight and a deformed phenotype, respectively.

### *2.5. Calculations and statistical analyses*

The cumulative nutrient release rate was calculated as percentage of total $NH_4^+$-N or P (mg) release in relation to the total N and P (mg) in the formulated fertilisers. The cumulative nutrient releases were applied to 13 non-linear models (Bock and Sikora, 1990) using nlsplot function in 'easynls' package (Emmanuel, 2017). Entry data were average of two replicates from the column perfusion test. The fitted models with lower residual sum of squares were selected to describe kinetics of $NH_4^+$-N and P released from different formulations. Based on the model selected, values for the predicted critical time (the time after which the quadratic relationship turned into linear (plateau) relationship) and the predicted maximum cumulative release were calculated. Mycorrhizal growth response (MGR) was calculated using the individual biomass data of 76R (mycorrhizal) plants and mean biomass of the *rmc* (non-mycorrhizal) plants from corresponding treatments (Eq. 1). Mycorrhizal N response (MNR) and mycorrhizal P response (MPR) were calculated in the same way, with values of total N and P uptake in plants, respectively. To determine whether mycorrhizal response means were significantly different from zero (in either a positive or negative direction), 95% confidence intervals (CI) were calculated and a treatment mean was deemed to be different where the 95% CI did not overlap zero.

$$\text{Mycorrhizal response (\%)} = \frac{\text{76R plant} - \text{mean } rmc \text{ plant}}{\text{mean } rmc \text{ plant}} \text{x } 100 \qquad (1)$$

A two-way ANOVA was performed on data for soil, plant and arbuscular mycorrhizal response at harvest with two experimental factors: tomato genotype (two) and formulation (six). Where ANOVA interactions and/or main effects were significant ($P \leq 0.05$), a Tukey's honestly significant difference (HSD) post hoc test was performed to identify differences between means among treatments with $\alpha$ level 0.05. Data analyses and principal component analysis (PCA) were performed in R statistical computing environment version 3.6.3 (R Core Team, 2020).

## 3. Results

### *3.1. Characterisation of formulated granular fertilisers*

Adjusting the ratio of chicken litter, MAP and urea altered the structure of the granular fertilisers (Fig. 1). The cross section of the 0:4 granules (i.e. containing only chicken litter) revealed a mixture of irregular shaped, <300 µm length, amorphous particles and an abundance of pores. The 1:3 and 2:2 formulations also had a coarse and porous structure. In contrast, the 3:1 formulation exhibited

a more compact structure. The 4:0 formulation and the MAP-only formulation produced a similar high P structure.

Results from elemental mapping of the granule cross sections by SEM-EDS are presented in Fig. 2. Whereas N was evenly distributed in the 4:0 formulation, it localised more on the boundary of the cross section of the MAP formulation. In contrast, N was not visible in the 0:4, 1:3, 2:2 and 3:1 formulations in this analysis. Phosphorus was more concentrated in the 4:0 and MAP formulations, than in the other formulations. Whereas P was distributed uniformly in the cross section of the 4:0 formulation, it was more localised to the centre and boundary of the MAP formulation, where it was associated with N. Phosphorus was distributed evenly in the 3:1 formulation, and unevenly distributed (P hotspots) in the other formulations that contained chicken litter.

Similar to the SEM-EDS images of N and P, the concentration of N and P increased in the granules where chicken litter was substituted with MAP (Table 1). In addition, the available N and P of those formulations also increased, compared to the 0:4 (chicken litter only) formulation. The available N was predominantly $NH_4^+$-N , compared to $NO_3^-$-N. Similarly, the proportion of water-extractable P increased as more MAP was substituted for chicken litter; for example, the water-extractable P was 34% and 83.2% in the 0:4 4:0 formulations, respectively. With the exception of the MAP formulation, which had a lower total N compared to total P (0.52 N/P ratio), all formulations were dominated by N, with N/P ratios between 2.73 and 3.39. Furthermore, the N/P ratio increased with increasing chicken litter. There was a significant difference in pH values of formulations with the lowest and highest values at 4.9 and 9.5 in the 4:0 and 0:4 formulations, respectively.

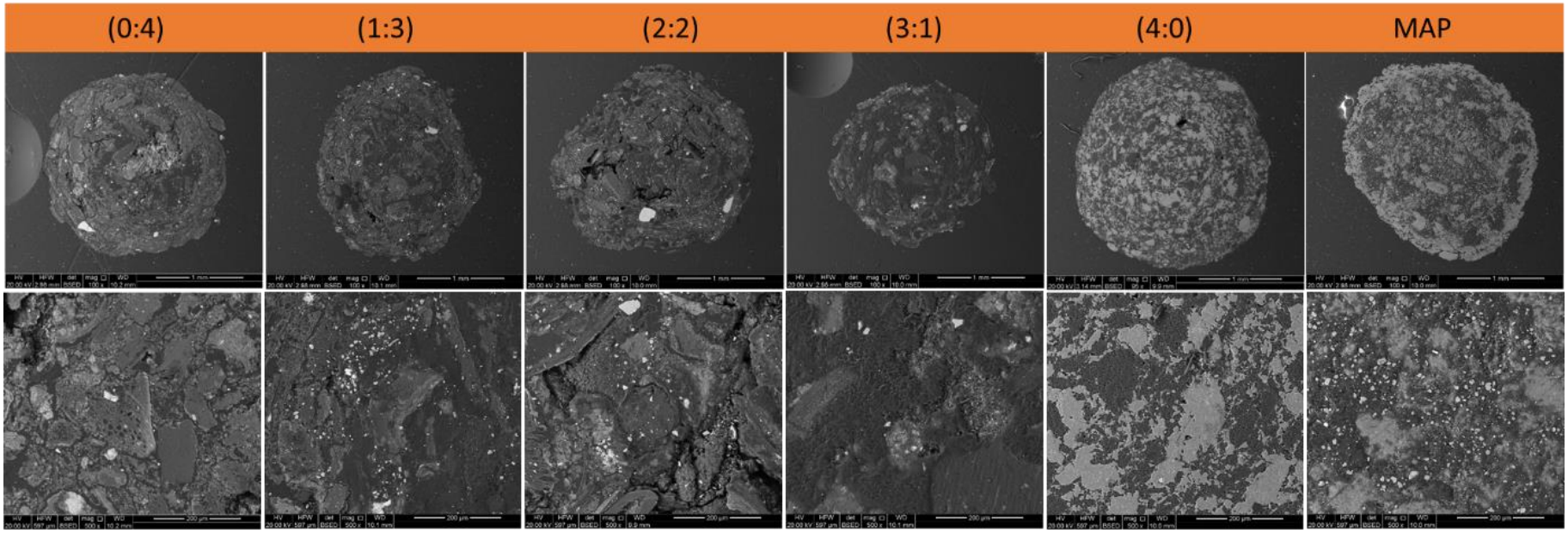



Figure 1. Backscattered-electron (BSE) images of granular fertilisers by scanning electron microscope (SEM) at 100x (top line) and 500x (bottom line). Scale bars shows 1 mm length. Photos are grouped vertically by formulation with values in parentheses the ratio of MAP:chicken litter.

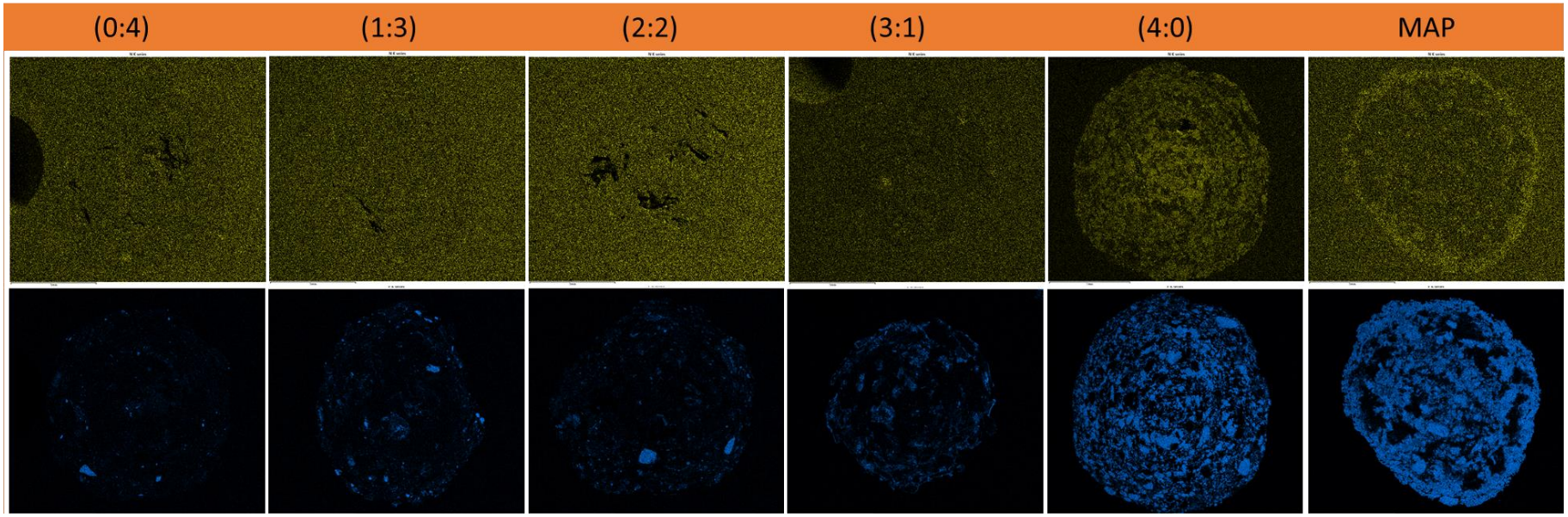



Figure 2. N distribution (top line) and P distribution (bottom line) in cross sections of granular fertilisers by SEM-EDX. Photos are grouped vertically by formulation with values in parentheses the ratio of MAP:chicken litter

Table 1. Chemical properties of formulated organo-mineral fertilisers. Values are mean (*n*=3).

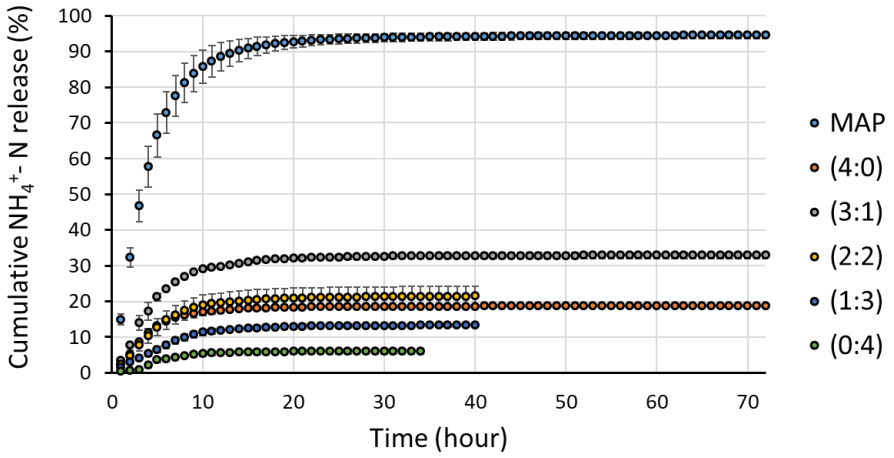
| Formulation (ratio MAP:chicken litter) | 0:4 | 1:3 | 2:2 | 3:1 | 4:0 | MAP |
|---|---|---|---|---|---|---|
| Total C (%) | 29.5 ± 0.15 | 28.6 ± 0.34 | 27.6 ± 0.1 | 28.6 ± 0.53 | 11.3 ± 0.25 | 0.6 ± 0.05 |
| C/N | 7.3 ± 0.04 | 5.5 ± 0.08 | 4.1 ± 0.08 | 2.3 ± 0.02 | 0.4± 0.13 | 0.1± 0.00 |
| Total N (%) | 4.0 ± 0.0 | 5.2 ± 0.06 | 6.8 ± 0.11 | 12.3 ± 0.28 | 28.9 ± 0.47 | 11.0 ± 0.05 |
| Available N (% total N) [(a)] | 2.2 ± 0.11 | 3.4 ± 0.08 | 3.9 ± 0.04 | 17.2 ± 0.65 | 17.0 ± 1.11 | 87.9 ± 1.38 |
| $NH_4^+$-N (mg/g) | 0.8 ± 0.04 | 1.7 ± 0.03 | 2.7 ± 0.09 | 21.1 ± 0.34 | 49.5 ± 2.04 | 98.3 ± 2.02 |
| $NO_3^-$-N (mg/g) | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| Total P (%) | 1.2 ± 0.01 | 1.6 ± 0.03 | 2.4 ± 0.03 | 4.4 ± 0.01 | 10.6 ± 0.06 | 21.1 ± 0.11 |
| Water extractable P (% total P) | 34.0 ± 0.63 | 42.7 ± 0.70 | 53.9 ± 0.58 | 58.7 ± 0.25 | 83.2 ± 2.33 | 74.6 ± 0.81 |
| Water-extractable P (mg/g) [(b)] | 4.0 ± 0.04 | 7.0 ± 0.06 | 13.0 ± 0.09 | 25.8 ± 0.16 | 87.5 ± 1.43 | 157.0 ± 1.36 |
| Acid-extractable P (mg/g)[(b)] | 9.9 ± 0.06 | 13.2 ± 0.16 | 20.6 ± 0.17 | 41.4 ± 0.33 | 102.6 ± 1.28 | 206.0 ± 1.29 |
| pH[(b)] | 9.5 ± 0.0 | 8.9 ± 0.01 | 8.1 ± 0.02 | 6.3 ± 0.01 | 4.9 ± 0.01 | 4.9 ± 0.02 |
| N/P | 3.39 ± 0.02 | 3.15 ± 0.04 | 2.80 ± 0.94 | 2.78 ± 0.06 | 2.73 ± 0.06 | 0.52 ± 0.01 |

[(a)]: Available N was measured as sum of $NH_4$-N and $NO_3$-N

[(b)]: Water/acid-extractable P and pH were measured following 16 hours sequential water-acid extraction of individual fertiliser granules (Tiessen and Moir, 1993).

### *3.2. Kinetics of N and P released from fertilisers*

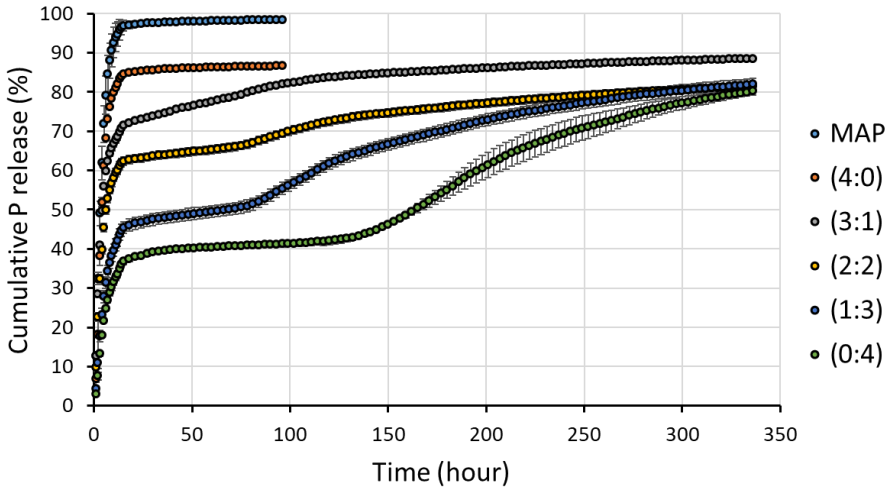
The kinetics of $NH_4^+$-N release was similar across the fertiliser formulations (Figure 3). For all formulations, the total amount of $NH_4^+$-N released over the course of the dissolution experiment was in proportion to the amount of total N content, except in the 4:0 formulation where the vast majority of N was added as urea. Cumulative $NH_4^+$-N release for the MAP and 4:0 formulations was 93.5% and 18.4%, respectively after 72 h; this was proportion to the initial total $NH_4^+$-N content in the formulations (compare Fig. 3 and Table 1). In contrast, the $NH_4^+$-N release over time of all other formulations was two to five times greater than their initial $NH_4^+$-N content (Fig. S2 and Table 1), indicating ammonification of organic N in the formulation. In addition, while the 4:0 and MAP formulation had a relatively similar predicted critical time for maximum cumulative $NH_4^+$-N release, the critical time for the other formulations containing chicken litter was slower by up to five hours compared to the 4:0 formulations.




| Formulation | Total N added (mg) | Predicted critical time release (hour) | Predicted maximum cumulative release (%) |
|---|---|---|---|
| MAP | 109.9 | 12.3 | 93.5 |
| (4:0) | 288.9 | 11.8 | 18.4 |
| (3:1) | 123.0 | 13.7 | 32.5 |
| (2:2) | 67.6 | 13.9 | 21.0 |
| (1:3) | 51.7 | 16.5 | 13.0 |
| (0:4) | 40.2 | 13.8 | 5.9 |

Figure 3. Kinetics of $NH_4^+$-N release in water (Graph) compared to total N added (Table) of fertilisers co-granulated with different ratios of MAP:chicken litter (ratios in parentheses). Values are means (*n*=2). Error bars show standard errors and where bars are not visible, their values are smaller than point size. N.B. Total N added refers to the amount of N added (in 1 g of granules) at the beginning of the dissolution experiment. The predicted critical time is the time after which the quadratic relationship turned into linear relationship (plateau).

In contrast to $NH_4^+$-N, all granular formulations had a similar predicted maximum cumulative P release, although P dissolution varied among the formulations (Fig. 4). In the first 20 hours, all formulations released P rapidly. Moreover, P release closely matched water-extractable P for all formulations (compare Fig. 4 and Table 1); for example, P dissolution increased as the ratio of MAP in the formulations increased and chicken litter decreased. For both the MAP and 4:0 formulations, maximum predicted cumulative P release (97% and 85%, respectively) was predicted to occur after 11 h, after which it remained stable. In contrast, for those formulations containing more chicken litter, P release continued to increase after 20 h. For example, about 85 % of cumulative P release was predicted after 22, 201 and 327 h. in the 3:1, 2:2 and 1:3 formulations, respectively. Similarly, for the 0:4 formulation, cumulative P release was predicted to be 88% of total P at 461 h.




| Formulation | Total P added (mg) | Predicted critical time release (hour) | Predicted max cumulative P release (%) |
|---|---|---|---|
| MAP | 226.2 | 11 | 97 |
| (4:0) | 110.4 | 11 | 85 |
| (3:1) | 45.0 | 22 | 85 |
| (2:2) | 23.5 | 201 | 79 |
| (1:3) | 15.5 | 327 | 81 |
| (0:4) | 12.1 | 18 | 40 |
| | | 461 | 88 |

Figure 4. Kinetics of P release in water (Graph) compared to total P added (Table) of fertilisers co-granulated with different ratios of MAP:chicken litter (ratios in parentheses). Values are means (*n*=2). Error bars show standard errors and where bars are not visible, their values are smaller than point size. N.B. Total P added refers to the amount of P added (in 1 g of granules) at the beginning of the dissolution experiment. The predicted critical time is the time after which the quadratic relationship turned into linear relationship (plateau).

### *3.3. Effects of fertilisers on plant growth and mycorrhizal colonisation*

Whereas plants supplied with MAP, 4:0 and 0:4 formulations had a similar shoot dry weight (SDW), those supplied with the other formulations had significantly higher SDW (Fig. 5a). Shoot N content was lowest in plants receiving MAP only (no additional urea), and increased as the amount of balancing urea in the formulation increased; specifically, 4:0 > 3:1 > 2:2 > 1:3 > 0:4 >MAP (Fig. 5b, Table S2). Whereas plant genotype did not have a main effect on shoot dry weight and shoot N content, shoot P content was affected by the interactive effects of formulation and genotype. In general, shoot P content in the 76R and *rmc* plants were lower in the 0:4 formulation compared to other formulations (Fig. 5c).

The formation of AM resulted in significantly different mycorrhizal growth response (MGR) with the application of the 0:4 and 3:1 formulations, all other formulations had a neutral impact on MGR. With the exception of the 0:4 formulation, all other formulations were associated with a negative mycorrhizal N response (MNR) (Fig. 5e). On the other hand, mycorrhizal P response (MPR) showed a more complex effect ranging from positive, through neutral, to negative among the

different formulations (Fig. 5f). Interestingly, mycorrhizal colonisation of roots decreased in a very consistent manner as the relative amount of chicken litter in the formulation decreased and urea addition increased (Fig. 6).

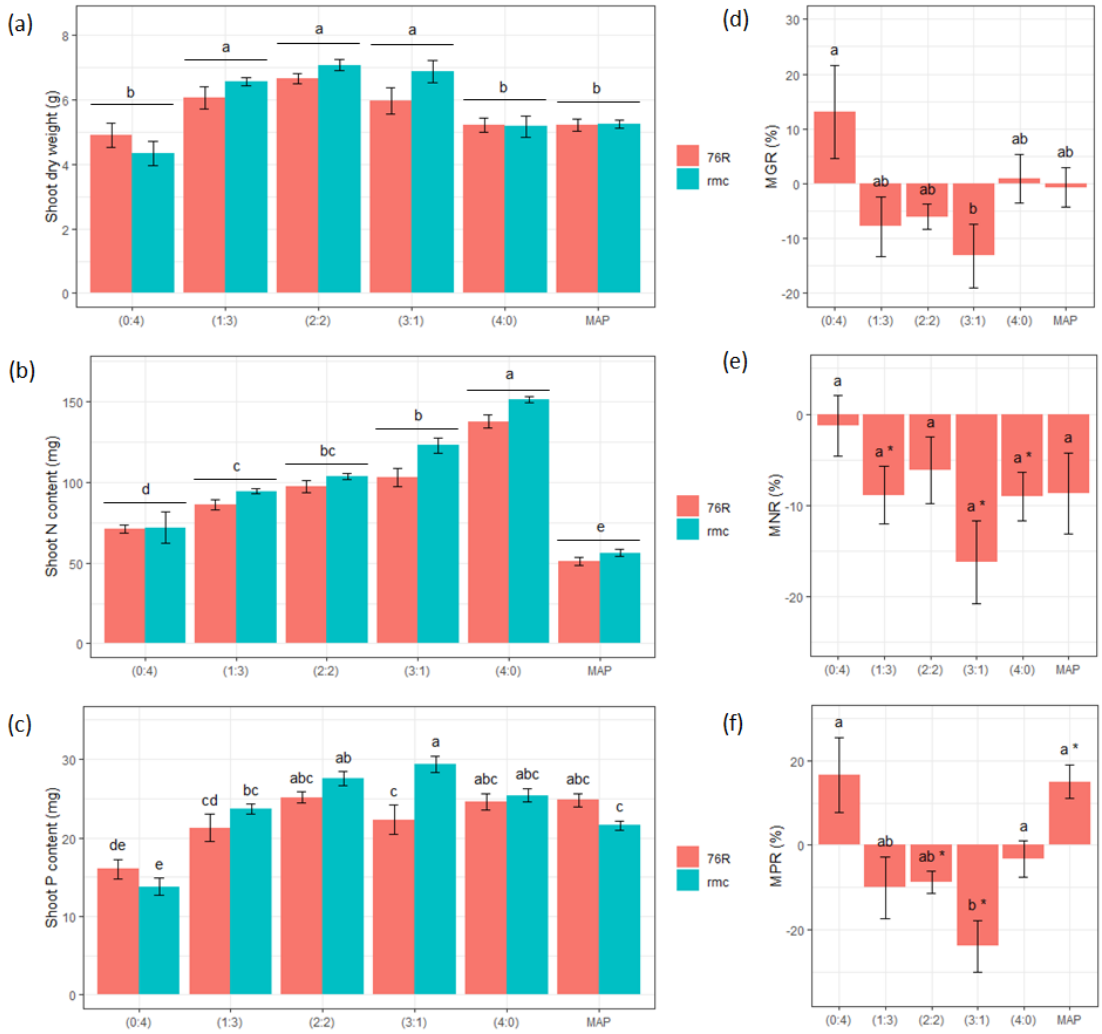



Figure 5. Shoot dry weights (a), shoot N contents (b) and shoot P contents (c) of 76R (mycorrhizal) and *rmc* (non-mycorrhizal) plants, mycorrhizal growth response MGR (d), mycorrhizal N response MNR (e) and mycorrhizal P response MPR (f) of 76R (mycorrhizal) plants grown with fertilisers co-granulated with different ratios of MAP:chicken litter (ratios in parentheses). Values are means ± SE, *n* = 5. Means with at least one common letter are not significantly different at the P≤0.05 level. Lines above bars indicate the main effect of fertiliser. Asterisks indicate means that are different from 0 at 95% CI.

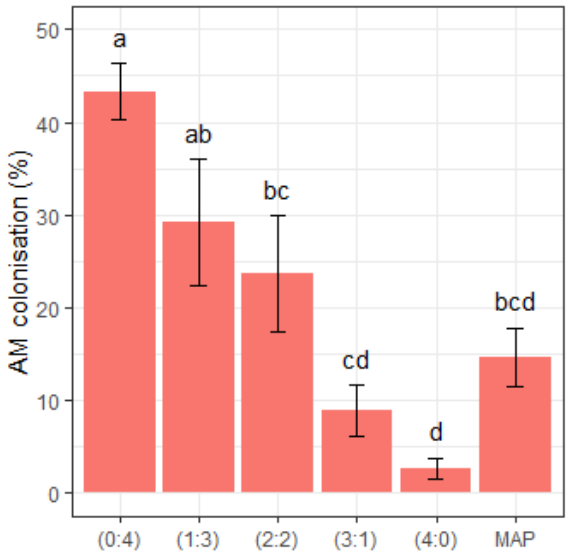



Figure 6. Mycorrhizal colonisation of roots of 76R (mycorrhizal) plants grown with six fertilisers co-granulated with varying ratios of MAP:chicken litter (values in parentheses). Means with at least one common letter are not significantly different at the P≤0.05 level.

## 4. Discussion

Granular fertilisers comprised of varying ratios of MAP and chicken litter (plus urea), that were suitable for practical field application (target granule diameter range of 2.00-2.36 mm) (Antille *et al.*, 2013) were successfully produced in the laboratory. The formulations containing chicken litter (0:4, 1:3 and 2:2) had a porous structure, with abundant pore spaces, likely due to the litter containing fragments of bedding material (straw). Interestingly, in the 3:1 formulation, which contained the smallest amount of chicken litter, the organic and mineral components were very closely associated likely because porosity filled by MAP and urea. The 4:0 formulation showed a slightly better P distribution through the granules when urea was added likely due to the higher solubility of urea in a water-based binder allowing “wetting” throughout the granule. For the practical application of these granules using current field equipment, it will be necessary to further test the compressive strength of these granules (Chen *et al.*, 2019; Mazeika *et al.*, 2016; Rodrigo Sakurada *et al.*, 2019). Analysis of the granules by SEM-EDS revealed differences in the spatial distribution of elements in the granules. These differences, such as on the surface and/or inside of the granules, may affect the dissolution kinetics of the different formulations (Ganetri *et al.*, 2021; Mazeika *et al.*, 2016). While SEM-EDS is a non-destructive analytical method for surface elemental analysis, it failed to detect N in some of the formulations; this is consistent with earlier work in which it was found that N at concentrations less than 10% (w/w) may not be observed by SEM-EDS (Makhlouf and Aliofkhazraei, 2015).

While the release of initial $NH_4^+$-N in the MAP and 4:0 formulations was quick and complete within 12 hours, $NH_4^+$-N release from the formulations containing chicken litter was slower, indicating a slow microbial hydrolysis and ammonification of urea and organic N components in the chicken litter (Sürmeli et al., 2017). Although the differences in predicted critical time for $NH_4^+$-N release were small (up to 5 hours), this suggests the materials containing chicken litter could behave as a 'slow release' fertiliser (Giroto *et al.*, 2017). Whereas urea was added to the formulations in an effort to equalise their N/P ratios, the N/P ratios of the granules decreased slightly as the amount of chicken litter was reduced. However, the N/P ratio was not different among formulations, except lower in the MAP formulation where no urea was added. The 4:0 formulation had a higher N/P ratio, but lower available N per unit weight and lower cumulative $NH_4^+$-N release compared to the MAP formulation, which was likely due to the fact that urea from 4:0 formulation released by dissolution but was not measured as $NH_4^+$-N. Furthermore, the addition of urea likely affected the pH of the formulations, however the effect on soil pH was small (Fig. S4). Nevertheless, given the effects of pH on soil nutrient availability (Barrow, 2017; Gustafsson *et al.*, 2012), it may be important to further investigate such effects in long-term field-based studies.

The dissolution of P in the MAP and 4:0 formulations was quick and complete. The predicted P release in the MAP formulation was faster than that in the 4:0 formulation, which is in contrast to previous research showing that P dissolution from MAP can be facilitated by co-application with urea, due to an increase in pH following urea hydrolysis (Hartikainen and Yli-Halla, 1996). However the effect observed in the present study could be due to slow hydrolysis of urea in the 4:0 formulation, under the dissolution test conditions used. In contrast, P dissolution in the granules containing chicken litter was relatively slow. Analysis of the granules by SEM-EDS revealed that P was localised in 'hotspots' in an organic matrix, which may have obstructed water penetration, thereby reducing the rate of dissolution. Additionally, the chicken litter had a high pH and contained significant amounts of Ca (Table S3), suggesting that the P in the litter may be Ca phosphates, thereby limiting dissolution. However, over time, the pH of the solution decreased (Fig. S5), which may lead to the dissolution of Ca phosphates, leading to P release. Finally, there may have also been some microbially-mediated mineralisation of organic P (e.g. phytate P) in the chicken litter-containing formulations (Mackay et al. 2017, Peirce et al., 2013). Taken together, these data indicated that formulated organomineral formulations had dual nutrient release properties, which were a function of the physical, chemical and biological characteristics of their constituent materials. Furthermore, the organomineral fertilisers produced using a sodium silicate based binder provided an additive benefit for environmentally 'friendly' fertilisers (Rajan and Kathirvel, 2021).

The results showed that all formulations were able to supply sufficient amounts of N and P , as seen in sufficient concentration of N and P in the shoots of the test plants (Reuter and Robinson,

1997), regardless their different rates of N and P release. As a result, plants receiving the MAP formulation had the lowest shoot N content, and plants receiving the 0:4 formulation had the lowest shoot P content, yet both had a shoot dry weight similar to that of plants receiving the 4:0 formulation which had the highest shoot N and P contents. This also suggests that N release and mineralisation in those formulations containing chicken litter, occurred rapidly. In contrast, shoot P contents were less variable among the different formulations, despite the considerable differences in rates of P release among the different formulations. That those products with the slow rate of P release resulted in the greatest plant growth, highlights the benefits (to plant growth) of a slow and sustained rate of P release. This supports a conclusion that the newly released P had a higher immediate bioavailability to plants (Peirce *et al.*, 2013)

High levels of AM colonisation were associated with formulations with slow N and P release, and low levels of colonisation were associated with formulations with fast N and P release. As all formulations were applied at a same total P rate, and all formulations had a similar cumulative P release, the results support the suggestion that slow release of P can support the formation of AM. In addition, the formulations that led to the highest and lowest levels of AM colonisation, also had lowest and highest amounts of urea added, respectively. It was noted, that the effect of the MAP formulation on AM colonisation did not follow the trends of N and P released, given that the total N provided from the MAP treatment was lower compared to other treatments. This is consistent with earlier work indicating that a high application rate of inorganic N and P fertilisers can retard formation of AM (Bücking and Kafle, 2015; Nouri et al., 2014), and that AM colonisation under high P conditions can be stimulated due to N limitation (Sylvia and Neal, 1990). Taken together, these data suggest that not only are the patterns of N and P release from the organomineral formulations better matched to a plant's demand over its life, but that the negative effects of high rates of inorganic N and P supply on the formation of AM may be reduced and/or avoided.

## Conclusions

Our results showed that whereas MAP increased the immediately available pool of nutrients in the soil, the addition of chicken litter delayed the release of nutrients. Importantly, the delay was well matched with plant nutrient demand, with plants receiving the organomineral formulations producing the greatest shoot dry weights. In addition, the rates of N and P release from the fertilisers were inversely related to levels of AM colonisation. Thus, the organomineral fertilisers produced here could have multiple benefits: reducing the use of synthetic fertilisers, making use of a waste material as a fertiliser, and providing patterns of nutrient supply that are more closely matched to plant demand.

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## Author Contributions

HTTN, MJM, SJWW and TRC conceptualized the study and designed experiments. HTTN and AP implemented experiments. HTTN and RB analysed data. HTTN wrote the manuscript. All the authors have read and edited the final manuscript.

## Data availability statement

Data sharing is not applicable to this article as all created data is already contained within this article or in the supplementary material.

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## Supplementary

Table S1. Powders of commercial MAP fertiliser (<250 µm) and chicken litter (<290 µm) were used to make organo-mineral fertilisers.

| | MAP powder | Chicken litter powder |
|---|---|---|
| Total C (%) | 0.58 ± 0.01 | 36.2 ± 0.74 |
| Total N (%) | 11.42 ± 0.12 | 4.4 ± 0.05 |
| Total P (%) | 21.68 ± 0.02 | 1.69 ± 0.01 |
| N/P | 0.53 | 2.60 |

Table S2. Proportion of MAP, chicken litter and urea in 100 g powder before granulating fertilisers

| Ingredient | (0:4) | (1:3) | (2:2) | (3:1) | (4:0) | MAP |
|---|---|---|---|---|---|---|
| MAP (g) | 0.00 | 2.47 | 6.75 | 15.99 | 50.54 | 100.00 |
| Chicken litter (g) | 100.0 | 95.11 | 86.64 | 68.36 | 0.00 | 0.00 |
| Urea (g) | 0.00 | 2.42 | 6.61 | 15.65 | 49.46 | 0.00 |

Table S3. Concentrations of micro- and macronutrients in fertilisers formulated with different ratios of MAP:chicken litter (ratios in parentheses). Values are means (*n*=3) (mg $kg^{-1}$).

| Formulation | K | Ca | Mg | Na | S | B | Cu | Zn | Mn | Fe | Al | Co | Mo |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| MAP | 1,839 | 1,414 | 7,777 | 1,214 | 10,958 | 57 | 2.9 | 65 | 343 | 11,219 | 11,002 | 3.6 | 14.3 |
| (4:0) | 814 | 735 | 3,965 | 622 | 5,491 | 31 | 4.9 | 37 | 172 | 5,717 | 5,481 | 1.9 | 7.2 |
| (3:1) | 15,326 | 19,017 | 4,633 | 3,657 | 5,216 | 37 | 92.3 | 316 | 377 | 2,256 | 1,893 | 1.8 | 9.3 |
| (2:2) | 16,144 | 18,893 | 4,199 | 29,780 | 4,300 | 34 | 96.0 | 305 | 344 | 1,203 | 940 | 1.4 | 8.1 |
| (1:3) | 15,930 | 19,280 | 3,987 | 27,893 | 3,997 | 33 | 100.3 | 316 | 347 | 837 | 558 | 1.4 | 7.9 |
| (0:4) | 16,638 | 20,646 | 3,750 | 40,275 | 3,807 | 34 | 102.8 | 332 | 348 | 552 | 342 | 1.3 | 7.6 |

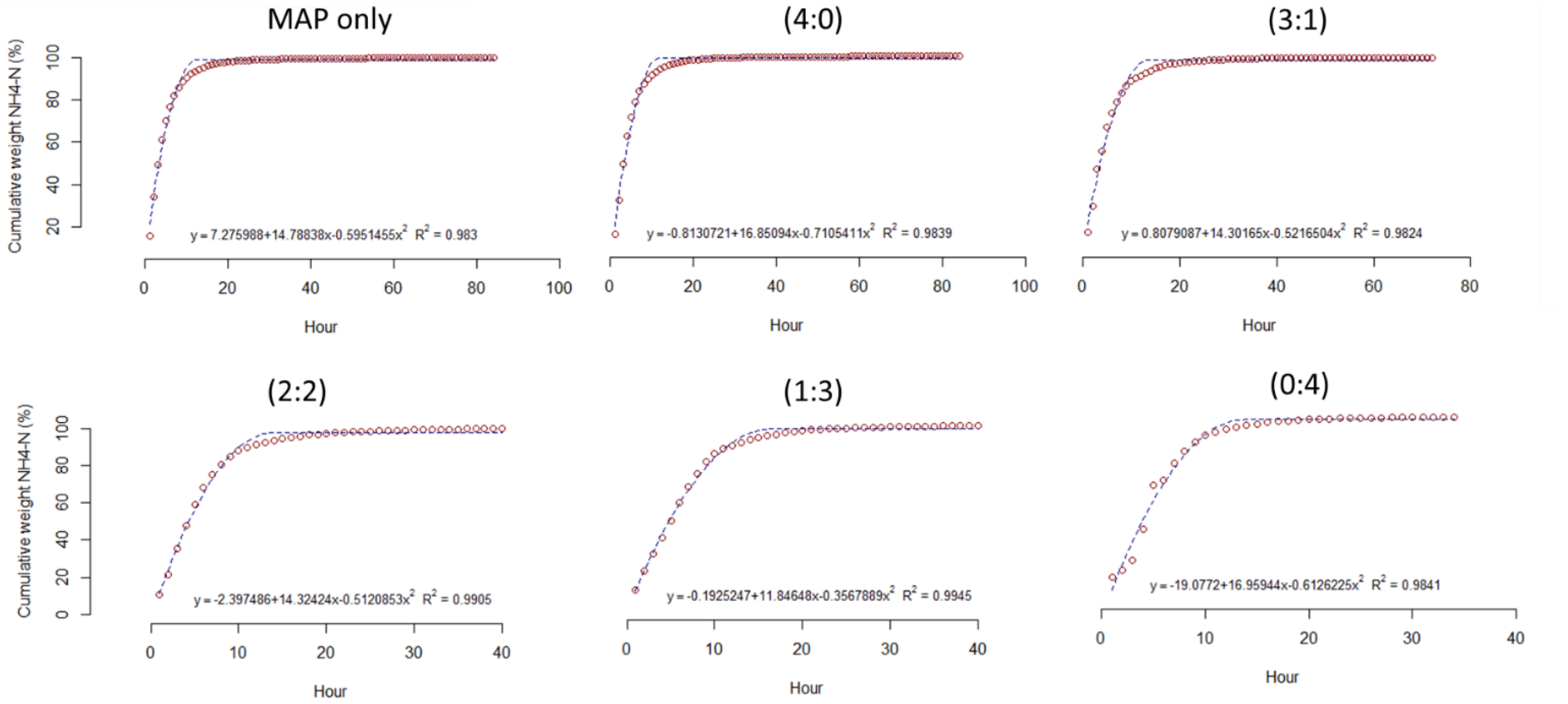
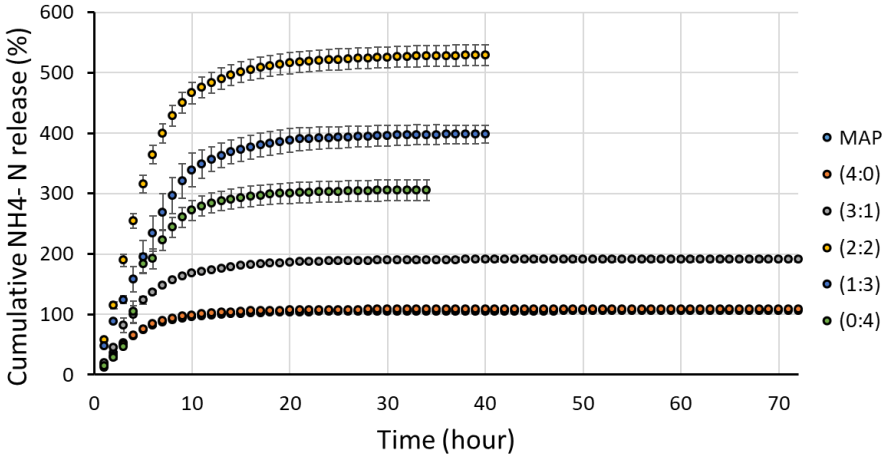



Figure S1. Kinetics of $NH_4^+$-N release from the fertilisers co-granulated with different ratios of MAP:chicken litter (ratios in parentheses) fitted by quadratic plateau model (values are means, n=2). Parameters and the coefficient of determination ($R^2$) of the model are estimated using quadratic linear model in R. Quadratic response model was defined by y ~ (a + b * X + c * I($x^2$)) * (x <= -0.5 * b/c) + (a + I(-$b^2$/(4 * c))) * (x > -0.5 * b/c). Where y is cumulative $NH_4^+$-N release (%), x is the hour from dissolution, a, b and c are parameters of the model. Predicted critical time for maximum cumulative $NH_4^+$-N release was defined by the value of x = -0.5 * b/c).




| Formulation | Innitial NH4-N (mg) | Predicted critical time release (hour) | Predicted maximum cumulative release (%) |
|---|---|---|---|
| MAP | 98.3 | 12.3 | 104.5 |
| (4:0) | 49.5 | 11.8 | 107.6 |
| (3:1) | 21.1 | 13.7 | 189.2 |
| (2:2) | 2.7 | 13.9 | 518.6 |
| (1:3) | 1.7 | 16.5 | 392.0 |
| (0:4) | 0.8 | 13.8 | 301.0 |

Figure S2. Kinetics of $NH_4$-N release in water (Graph) compared to initial $NH_4$-N content of fertilisers co-granulated with different ratios of MAP:chicken litter (ratios in parentheses). Values are means (*n*=2). Error bars show standard errors and where bars are not visible, their values are smaller than point size. N.B. Total P added refers to the amount of P added (in 1 g of granules) at the beginning of the dissolution experiment. The predicted critical time is the time after which the quadratic relationship turned into linear (plateau) relationship.

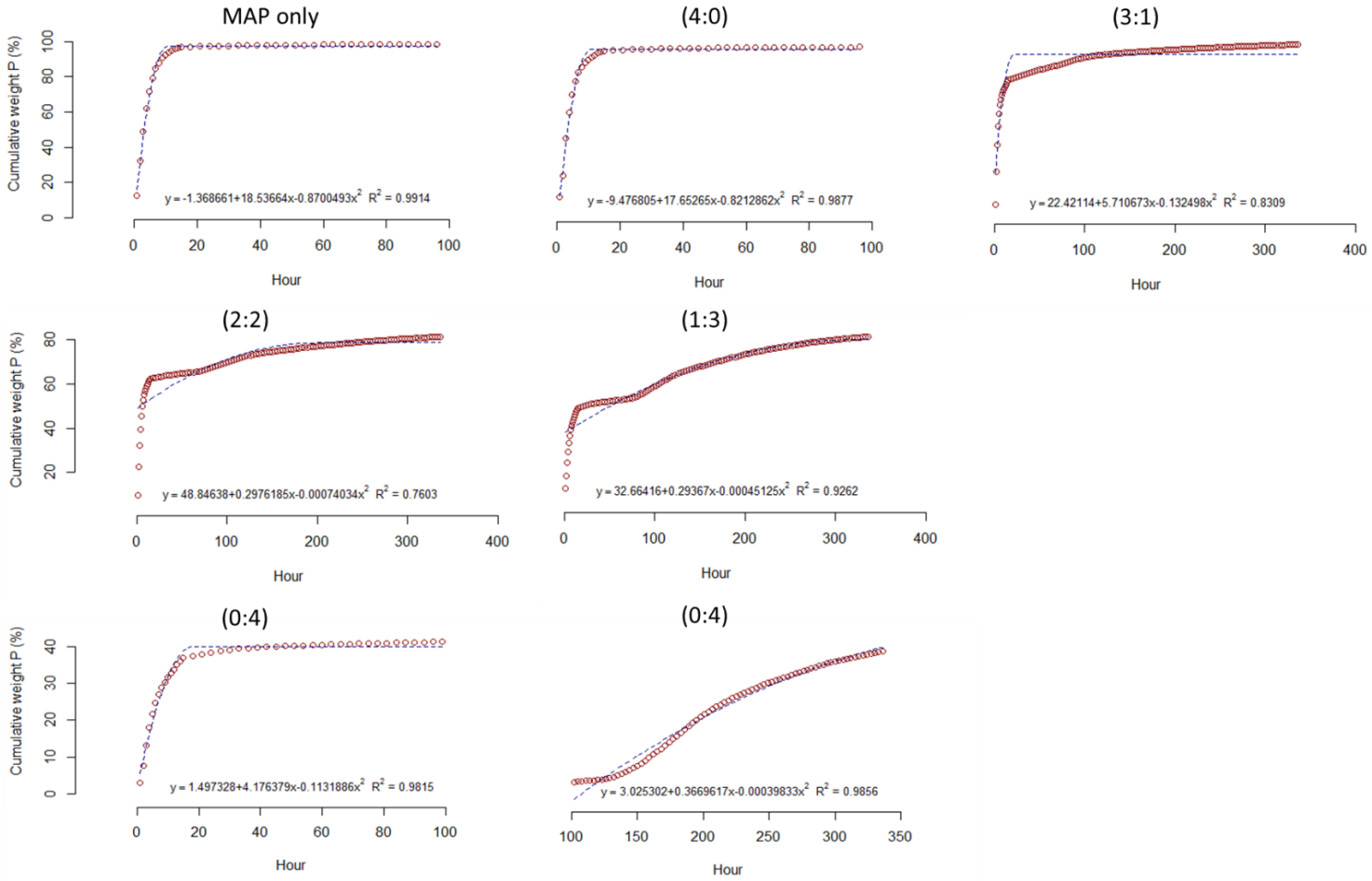



Figure S3. Kinetics of P release from the fertilisers co-granulated with different ratios of MAP:chicken litter (ratios in parentheses) fitted by quadratic plateau model (values are means, n=2). Parameters and the coefficient of determination ($R^2$) of the model are estimated using quadratic linear model in R. Quadratic response model was defined by y ~ (a + b * X + c * I($x^2$)) * (x <= -0.5 * b/c) + (a + I(-$b^2$/(4 * c))) * (x > -0.5 * b/c). Where y is cumulative P release (%), x is the hour from dissolution, a, b and c are parameters of the model. Predicted critical time for maximum cumulative P release was defined by the value of x = -0.5 * b/c).


Figure S4. Soil pH at harvest from the fertilisers co-granulated with different ratios of MAP:chicken litter (ratios in parentheses).

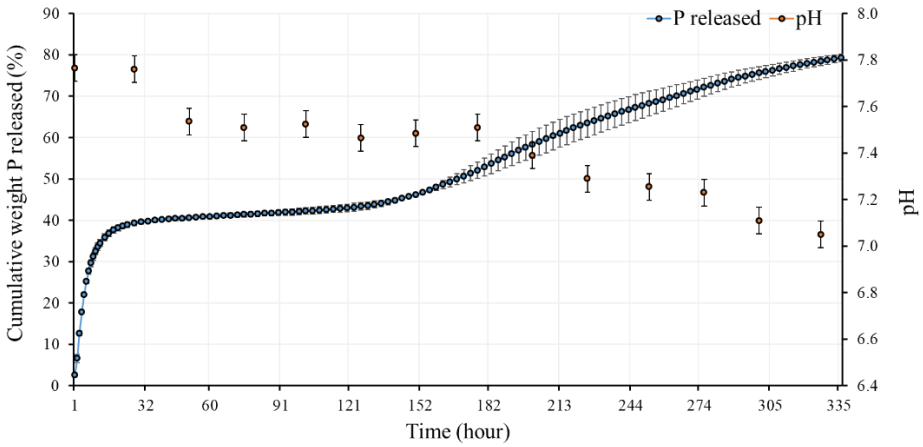



Figure S5. Kinetics of P release in relation to pH of the 0:4 (chicken litter only) fertiliser.

# CHAPTER 6: GENERAL DISCUSSION

## 1. General discussion

As highlighted at the beginning of this thesis, there is an urgent need to improve P use efficiency in agricultural production, which could occur through reducing our reliance on inorganic P resources while also increasing the recycling of P from agricultural wastes. The rapid release of P from inorganic P fertilisers contributes to low P use efficiency. In addition, high available P in soils may not benefit plant P uptake and may contribute to environmental pollution. Thus, combining inorganic and organic P sources for fertiliser purposes is considered a potential solution for efficiently using P resources for crop production.

The work presented in this thesis aimed to understand how plants respond to the combined use of inorganic P and organic amendments as fertilisers, and, further, it investigated how the fertilisers interact with the arbuscular mycorrhizal symbiosis.

Across four independent experiments, I examined the responses of 76R (mycorrhizal) and *rmc* (non-mycorrhizal) tomato plants to different P sources (inorganic, organic and mixed). In these experiments, the inorganic P source was phosphoric acid (Chapter 2- 4), MAP and/or MAP combined urea (indicated as (4:0)) (Chapter 5); the organic P source was unground (Chapter 2- 4) or ground chicken litter (Chapter 5). While I included a control without P added to soil in all experiments, plant growth responded very positively to P addition in each experiment. Thus, the P addition control was excluded in all data analyses. This meant I focused on the comparison of the responses of the tomato plants in mixed P sources to solely inorganic and solely organic P sources. Furthermore, based on P application rate, and effects on plant growth at the time of harvest, I categorise my experimental design following different nutrient input scenarios. Briefly, the nutrient inputs were low N and low P (Chapter 2), low N and low to high P (Chapter 3), low and high N and P (Chapter 4), and high N and high P (Chapter 5).

I found that where total P application rates were matched between different sources of P fertiliser materials, the solely inorganic P source provided higher readily available P in soils compared to the mixed source and the solely organic P source (Chapter 2- 4). However, the higher available P concentration in the soil obtained after applying inorganic P source did not translate into greater shoot P content compared to the mixed P source (Chapter 2, 3 and 5). In contrast, P concentration remained high in the soil at the time of harvest (Chapter 2, 3 and 5) indicating that fast rate of P release did not support overall plant P uptake, which may be caused by strong P sorption reactions (Holford, 1997) or plant-soil microbe P completion (Clausing and Polle, 2020). Indeed, higher readily available P concentration in the solely inorganic P source resulted in higher shoot growth early in the plants development (Chapter 4), whereas the application of mixed P sources enabled plants to gain a final growth and nutritional advantage (Chapter 2- 5). This indicated that the tomato plants required sufficient P supply from the combined inorganic and

organic P sources. In addition, to trial the effect of mixed P sources in an applied sense, I formulated granular organomineral fertilisers combining MAP and chicken litter (Chapter 5). I found that, P source affected the structure of granules and kinetics of $NH_4^+$-N and P release from granular fertilisers. Consequently, the organomineral formulations had dual nutrient release characteristics that were more closely matched with plant nutrient demand, and thus increased plant growth, while not acting antagonistically to AM colonisation.

Arbuscular mycorrhizal colonisation was affected by P application rate, but not P source (Chapter 2 and 3). This could be because of the same P application rates, the solely inorganic P, the solely organic P and the combined P sources produced soil P concentration that were lower than a threshold level that could affect AM colonisation (Bolan *et al.*, 1984; Shukla *et al.*, 2012). However, there was a case that AM colonisation was varied in different fertiliser formulations (Chapter 5), which could be due to the effect of soil N:P stoichiometry on controlling the effect of P addition on AM colonisation (Pan *et al.*, 2020), as all formulations were different in the concentration of N and P after adding urea.

In contrast to AM formation, P sources affected growth, P and N nutrition of tomato mycorrhizal plants compared to non-mycorrhizal plants. In all cases mycorrhizal and non-mycorrhizal plants responded equally in the soil where the mixed P source was added (Chapter 2-5). However, the solely inorganic P source consistently favoured AM plants across low to high P application rates, whereas AM plants performed less well than non-AM plants in the solely organic P source (Chapter 3). While the trend of AM responses to different P sources were different in Chapter 2, 4 and 5, compared to in Chapter 3, I will discuss possible reasons below with a focus on the AM responses (MGR, MNR and MPR) of tomato shoots, in relation to nutrient characteristics of P sources and shoot nutrient status.

Over three experiments (Chapter 2, 3 and 5), there was a continuum of mycorrhizal responses from positive, neutral, to negative, in shoots of tomato 76R plants. It should be noted that from changes in nutrient inputs, a wide range of soil pH (5.3 to 7.1) was observed over the three experiments (Table S1), likely due to the use of phosphoric acid as the inorganic P source (Chapter 2 and 3) and addition of urea to balance N/P ratio (Chapter 5). However, the change in soil pH appears to have had minimal impact on mycorrhizal responses (Fig. S1).

With respect to the nutrient characteristics of the different P sources, the results showed that positive mycorrhizal responses occurred where inorganic P was the primary fertiliser input (Chapter 2 and 3), and where MAP, or organic P, was the primary input (Chapter 5). The commonality across these experiments was low initial available N in the applied materials. The application of the solely inorganic P source in Chapters 2 and 3 was associated with a low available N input, as phosphoric acid does not have native N, whereas chicken litter does. The application of

MAP and solely organic P source in Chapter 5 also resulted in low available N inputs, as the formulations had low N/P ratios and did not have urea addition, respectively. Thus, the results suggested that low available N input or slow release N, may be an important factor in favour of positive mycorrhizal responses.

At the time of harvest, the tomato plants had different N and P nutrient statuses, which could be represented by the different N/P ratio of shoots, approximately 20 (Chapter 2) and less than 10 (Chapter 3 and 5). I considered shoots with N/P ratios of between 10 - 20 as either N or P limited (limited NP), and less than 10 as N limited, based on Yan *et al.* (2017). In addition, while the plants in Chapters 3 and 5 had N limited shoots, their low N/P ratios were due to low N inputs and high P inputs, respectively. Figure 1 shows the distribution of shoots with different nutrient status and mycorrhizal response. Tomato plants did not exhibit positive mycorrhizal responses in shoots that had high P in relation to N. In addition, where shoot N was limited, positive mycorrhizal responses were more pronounced in plants receiving low N inputs, rather than low P inputs. In other words, the results suggest that the status of N/P in plant shoots (as affected by nutrient inputs) may be an important driver of positive mycorrhizal responses. Together, with the nutritional characteristics of P resources, it may be informative to apply a systematic variation method (Beauchamp and Hamilton, 1970; Homes, 1963) that permits an estimation of the optimal N/P ratio of nutrient inputs, to maximise yields and AM responses. Where AM responses are improved, it could help to reduce reliance on fertiliser inputs, while maintaining crop yields.

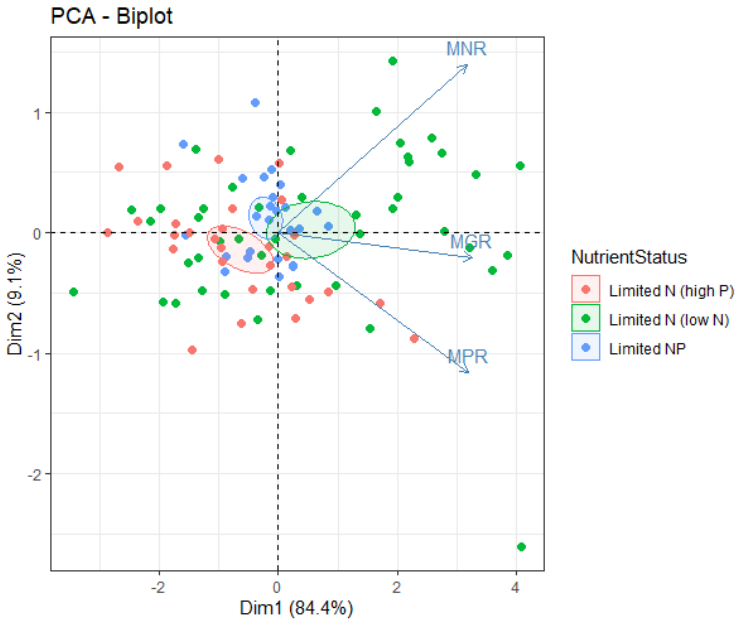



Figure 1. Principal component analysis (PCA) of functional traits of tomato 76R (mycorrhizal) plants based on nutrient status of shoots. Displayed traits include shoot mycorrhizal growth response (MGR), shoot mycorrhizal N response (MNR), and shoot mycorrhizal P response (MPR). Shoots had N/P between 10 and 20 (Limited NP, Chapter 2). Shoots had N/P less than 10 and low N input (Limited N (low N), Chapter 3). Shoots had N/P ratio less than 10 and high P input (Limited N (high P), Chapter 5).

Finally, the impact of forming AM associations on plant growth changed over time, as indicated in the results of Chapter 4 experiment. That the positive effects of forming AM on plant biomass had appeared in early plant growth, highlights the importance of AMF on supporting plant P acquisition where plant roots had not fully developed for P scavenging. However, as the early positive effects of forming AM on plant biomass had disappeared by the time of harvest, the contribution of AMF to plant growth and nutrition may be underestimated. Thus, studies of AM impacts on plants, which rely on a single endpoint analysis of plant biomass, which is a common experimental approach for obvious practical reasons, can lead to different interpretations of treatment effects, depending on when the final harvest is performed. This finding is echoed by other studies using high-throughput phenotyping (Riley *et al.*, 2019; Tran *et al.*, 2020; Watts-Williams *et al.*, 2019), and highlights the power of this approach. This may be especially important where there is a need to understand how different plant growth stages are affected by AM, and indeed other factors that may alter plant growth and development (e.g., nutrient, heat, salinity and drought).

## 2. Conclusions

The results presented in this thesis demonstrate that the combined use of inorganic and organic P sources were able to maintain available nutrients in the soils that helped to increase plant growth. Importantly, the organomineral fertilisers generated from a P-rich waste material had an advantage compared to single P sources, in terms of impacts on plant growth and nutrition. Thus, blending inorganic and organic P sources could be used as alternative P fertilisers for plants. Such an approach has the potential to benefit sustainable agricultural development via reducing the use of chemical P fertilisers, improving P use efficiency, and indirectly supporting soil health via fostering the formation of AM. Given the complex interactions between N and P observed here, and their apparent impacts on AM responses, there is a need for further investigations of this nature. As was the case here, such investigations will benefit from the application of high-throughput phenotyping approaches, and could be used to inform the further development of fertiliser products.

## 3. Future directions

Based on the outcomes of my research, I have identified a number of areas that I believe should be the focus of future investigations. These include:

- Formulation of organomineral fertilisers that incorporate mineral P with a wider range of P-rich organic materials, such as pig manure and mushroom compost.

- Formulation of organomineral fertilisers considering the effect of N and P materials in terms of forms and ratios. For example, using slow release N materials and designing fertilisers with optimal N/P ratios (e.g., applying a systematic variation method).
- Optimisation of organomineral formulations that have standard physical characteristics, which allow them to be used with available farm machinery. For example, consideration of the particle size of the organic material, and binding agents for granulating.
- Testing the effects of different organomineral fertilisers on various soil types in the field, and with a range of different crop species.
- Implementing life cycle analysis to explore economic benefits of organomineral fertilisers. Such an analysis will need to consider factors such as energy use efficiency, transportation, infrastructure needs, cost-effective investment, and potential environmental impacts.

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## Supplementary

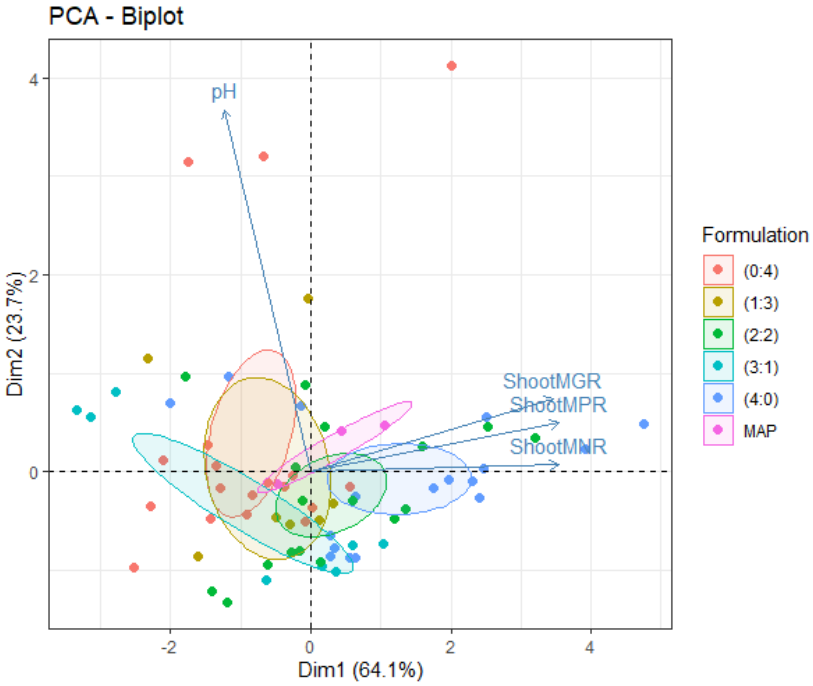


Figure S1. Principal component analysis (PCA) of functional traits of tomato 76R (mycorrhizal) plants grown with different P source ratios of MAP:chicken litter (values in parentheses). Displayed traits include shoot MGR, shoot MNR, and shoot MPR. Data were compiled from Chapter 2, 3 and 5.