The influence of phosphorus fertiliser addition on soybean nitrogen fixation and yield

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Declaration

This thesis has not been submitted in support of an application for another degree at this or any other university. It is the result of my own work and includes nothing that is the outcome of work done in collaboration except where specifically indicated.

Hannah Josephine Walling
Lancaster University, March 2025

Statement of Authorship

This thesis is prepared in the alternative format as a series of four papers presented in Chapters two – five, with some intended for submission to peer-reviewed journals or currently under review. These chapters have co-authors in addition to my supervisory team at Lancaster University, the contributions of all co-authors is set out below using the CRediT taxonomy. Chapters 1 and 6 are introductory and discussion chapters and are not intended for submission. In addition, three Appendixes are included containing supplementary materials, extended methods and results, and additional preliminary experimental work.

Chapter two – *in review – Field Crops Research*

Hannah J. Walling, José L. Rotundo, Lucas Borrás, Philip M. Haygarth, John N. Quinton, Shane A. Rothwell and Mariana C. Rufino. **Influence of environmental and management factors on the effectiveness of phosphorus fertiliser in improving soybean yield: a global meta-analysis**

Hannah J. Walling: conceptualisation, methodology, formal analysis, writing – original draft preparation, José L. Rotundo: methodology, conceptualisation, writing-review and editing, Lucas Borrás: conceptualisation, writing-review and editing, Philip M. Haygarth: conceptualisation, supervision, writing-review and editing, John N. Quinton: supervision, writing-review and editing, Shane A. Rothwell: supervision, writing-review and editing, Mariana C. Rufino: methodology, conceptualisation, supervision, writing-review and editing, funding acquisition.

Chapter three – *in preparation – intended for publication as a Letter to Editor and has been presented as such.*

Hannah J. Walling, Shane A. Rothwell, Moritz Reckling, Philip M. Haygarth, John N. Quinton and Mariana C. Rufino. **Potential limitations to the quantification of relative abundance of ureides to assess soybean symbiotic nitrogen fixation**

Hannah J. Walling: conceptualisation, investigation, methodology, formal analysis, writing – original draft preparation, Shane A. Rothwell: supervision, methodology, writing-review and editing, Moritz Reckling: methodology, investigation, Philip M. Haygarth: conceptualisation, supervision, writing-review and editing, John N. Quinton: supervision, writing-review and editing, Mariana C. Rufino: methodology, conceptualisation, supervision, writing-review and editing, funding acquisition.

Chapter four – *in preparation*

Hannah J. Walling, Shane A. Rothwell, José L. Rotundo, Lucas Borrás, Philip M. Haygarth, John N. Quinton, Mariana C. Rufino. **Influence of phosphorus fertiliser on soybean nitrogen fixation and yield**

Hannah J. Walling: conceptualisation, investigation, methodology, formal analysis, writing – original draft preparation, Shane A. Rothwell: supervision, methodology, writing-review and editing, José L. Rotundo: conceptualisation, writing-review and editing, Lucas Borrás: conceptualisation, writing-review and editing, Philip M. Haygarth: conceptualisation, supervision, writing-review and editing, John N. Quinton: supervision, writing-review and editing, Mariana C. Rufino: methodology, conceptualisation, supervision, writing-review and editing, funding acquisition.

Chapter five – *in preparation*

Hannah J. Walling, Shane A. Rothwell, José L. Rotundo, Lucas Borrás, Philip M. Haygarth, John N. Quinton, Mariana C. Rufino. **Phosphorus partitioning and mobilisation within soybean under contrasting phosphorus fertiliser treatments**

Hannah J. Walling: conceptualisation, investigation, methodology, formal analysis, writing – original draft preparation, Shane A. Rothwell: supervision, methodology, writing-review and editing, José L. Rotundo: conceptualisation, writing-review and editing, Lucas Borrás: conceptualisation, writing-review and editing, Philip M. Haygarth: conceptualisation, supervision, writing-review and editing, John N. Quinton: supervision, writing-review and editing, Mariana C. Rufino: methodology, conceptualisation, supervision, writing-review and editing, funding acquisition.

Abstract

This thesis tests the hypothesis that phosphorus (P) fertiliser addition can optimise nitrogen (N_2) fixation processes and increase soybean yield. Using a combination of controlled environment studies, field trial and global meta-analysis, this thesis aids in achieving sustainable soybean production through building improved understanding of the effects of P fertiliser addition to inform future fertiliser guidelines, crop models and management practices.

Global meta-analysis showed an increase in soybean response to P fertiliser addition with seed yield increasing by 25%. This also highlighted the complexity of soybean yield response to P fertiliser, with a several key management and environmental conditions having a significant effect, including soil P concentration, pH, fertiliser type and rate of application and climatic conditions – indicating soybean yield cannot be increased by single P fertiliser applications alone.

Controlled environment studies revealed P addition significantly increased nitrogen (N_2) fixation. Key nodule traits significantly correlated with shoot N; however, further work should examine the mechanistic pathways driving the increase in nodule formation. Interestingly, controlled environment studies revealed nodule function was not influenced by P fertiliser addition. Instead, regulatory mechanisms such as maintenance of nodule P concentration and leghaemoglobin concentration under low P conditions maintained N_2 fixation. Through combined analysis of multiple growth parameters and measures of plant physiology, seed yield was found to increase under P fertiliser addition. Seed P concentration also increased following P fertiliser addition.

Results of this thesis contribute to our understanding of soybean response to P fertiliser addition, particularly the improvement to key nodule traits to improve N_2 fixation and the partitioning and remobilisation of resources to improve yield. This now needs to be upscaled at differing environmental and management conditions and incorporated into crop models to ensure the sustainable use of P fertiliser in soybean production globally.

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night activities and the world's worst Christmas meal). To the girls of LG505, thanks for making early PhD days spent in the basement enjoyable, with our trip to Lake Como being a highlight.

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List of Abbreviations and Acronyms

°C degree celsius (unit of temperature)
µl microlitre
µMol micromole (molar concentration)
ANCOVA analysis of covariance (statistical test)
ANOVA analysis of variance (statistical test)
ATP Adenosine triphosphate
B boron
C carbon
Ca calcium
CE controlled environment
CFU Colony forming unit (measure of viable colonogenic cell numbers (CFU/ ml)
cm centimetre
CO_2 carbon dioxide
Cu copper
CV coefficient of variation
df degrees of freedom
DI deionised [water]
FAO food and agriculture organisation
Fe iron
g gram
H_2SO_4 sulphuric acid
ha hectare
HNO_3 nitric acid
ICP-OES Inductively coupled plasma optical emission spectroscopy
k effect sizes
K potassium
KCl potassium chloride
kg kilogram
kt kilotonnes (10^3 tonnes)
l litre
lm (linear model)
LoD Limit of detection
lr log transformed ratio of means
m metre
M mole (molar concentration)
MAG Modified Arabinose Gluconate (growth medium)
Mg magnesium
mg milligram
Mha megahectares (10^6 hectares)
ml millilitre
mm millimitre
MPa megapascal (10^6 pascals)
mse mean squared error
Mt megatonnes (10^6 tonnes)

N nitrogen
n sample size
N_2O Nitrous oxide
NA natural abundance
NADPH nicotinamide adenine dinucleotide phosphate
$NaHCO_3$ sodium bicarbonate
NaOH sodium hydroxide
Ndfa nitrogen derived from fixation
NH_3 ammonia
nm nanometre (unit of length, specifying wavelength of light)
NO_3^- nitrate
O_2 oxygen
P phosphorus
p probability value
P_2O_5 phosphate
pH potential for hydrogen
ppm parts per million
Pseudo R^2 proportion of variance explained by model (calculated using the function $R^2=(QT-QE)/QT$)
QE Cochran's Q-test statistic (test to assess between response heterogeneity)
QM Q-test statistic for moderators (test of the ability of moderators to account for a significant proportion of the variability)
R1 Beginning of flowering (phase of phenological development)
R^2 correlation coefficient
R2 Full flowering (phase of phenological development)
R3 Beginning of pod formation (phase of phenological development)
R5 Beginning of seed filling (phase of phenological development)
R7 Beginning of full maturity (phase of phenological development)
RAU abundance of relative ureides
RAU relative abundance of ureide
rpm revolutions per minute (unit of rotational speed)
S sulphur
SD standard deviation
SD standard deviation
SE standard error
USDA United States Department of Agriculture
ZALF Leibniz Centre for Agricultural Landscape Research
Zn zinc
$\delta^{15}N$ delta-15 Nitrogen (heavy nitrogen isotope)

1. General Introduction

The need to increase food productivity whilst minimising economic and environmental costs remains one of the largest problems of modern agricultural production. The high protein content of soybean coupled with its ability to fix atmospheric nitrogen gas (N_2) makes it both a highly useful yet highly intensive crop, often requiring large amounts of additional resources (such as phosphorus (P) fertiliser) to meet the production demands. This thesis hopes to address the problem of closing soybean yield gaps through improving the efficiency of P fertiliser use in soybean production. This brief introduction aims to provide information on the wider context before defining the specific aims and hypothesis addressed through this thesis.

1.1. Soybean

1.1.1. Soybean production

Soybean (*Glycine max* L. (Merr.)) is the fourth largest field crop by volume (representing 61% of global legume production) and the largest source of both vegetable oil and animal protein feed (Herridge et al., 2008b; Masuda and Goldsmith, 2009; Pagano and Miransari, 2016). The high protein content of soybean seed (approximately 40%) and the high oil content (approximately 20%) rich in essential fatty acids such as poly-unsaturated fatty acid, linoleic acid and linolenic acid aids in its popularity for production (Balboa et al., 2018; Messina, 1999; Riskin et al., 2013). Soybean production in 2023 was approximately 371.18 Mt, with 83% of that being produced in the top four soybean producers Brazil, United States of America (U.S.), Argentina and China (FAOSTAT, 2023). The top ten soybean producers (Table 1.1) account for 87.5% of soybean produced globally.

Table 1.1. Soybean domestic production (Mt), area (Mha) and yield (kg ha^{-1}) of the top ten soybean producing countries with the global ranking for yield and area shown in brackets, using FAO statistical data for 2023 (FAOSTAT).

Country	Production (Mt)	Area (Mha)	Yield (kg ha^{-1})
Brazil	152.1	44.4 (1)	3423.0 (3)
U.S.	113.3	33.3 (2)	3398.7 (4)
Argentina	25.0	14.4 (3)	1744.5 (7)
China	19.5	10.0 (5)	1952.6 (14)
India	15.0	13.1 (4)	1145.3 (20)
Paraguay	10.2	3.6 (6)	2826.2 (40)
Canada	7.0	2.3 (8)	3087.1 (43)
Russia	6.6	3.5 (7)	1885.7 (46)
Ukraine	4.7	1.8 (9)	2585.9 (55)
Bolivia	3.7	1.8 (10)	2012.3 (76)

Within the top ten soybean producers, large variability can be observed in yield values and global yield ranking compared to other producers in 2023 (Table 1.1). For example, Argentina,

despite being ranked third in terms of global production, had an average yield approximately 50% smaller than that of Brazil and the U.S., ranking it 7th globally. The yield potential of a crop is the yield when grown with non-limited water and nutrients and the effective control of biotic stress. The yield gap, known as the difference between the yield potential (yield when grown under non-limited water and nutrient conditions with effective control of biotic stress) and actual yield achieved, is used to assess how management options can increase crop productivity (Edreira et al., 2017). Soybean yield gaps under rainfed conditions in Brazil, Argentina and the United States have been estimated to be 22, 42 and 32% respectively (Di Mauro et al., 2018; Merlos et al., 2015; Sentelhas et al., 2015).

1.1.2. Soybean nitrogen fixation

Despite atmospheric nitrogen gas (N_2) being 78% the earth's atmosphere, it is not readily available to plants unless the covalent bond between atoms can be broken to produce ammonia (NH_3) or nitrate (NO_3^-) (Wang, 2024). This process can only be achieved through naturally occurring N_2 fixation or the industrial Haber-Bosch process. The production and use of synthetic N fertiliser through the Harber-Bosch process has multiple environmental impacts, from the use of fossil fuels to power the process of production to the release of nitrous oxide (N_2O) gas after application in the field (Gao et al., 2023). With the expected rise of fossil fuels, the high economic costs of synthetic N fertilisers are set to increase further, reducing profit margins and possibly becoming unaffordable to small-scale farmers in developing regions. Alongside this the price of synthetic N fertilisers shows patterns of great fluctuations over time (Figure 1.1), closely tracking volatile international fossil fuel supplies and prices (Snapp et al., 2023).

The alternative production of plant available NH_3 can be achieved through the process of biological N_2 fixation. The high seed protein content of legumes requires high N uptake, with lack of N being the primarily limiting resource for soybean growth and yield after water (Giller and Cadisch, 1995; Hirel et al., 2007; Sinclair and Wit, 1975). Whilst legume species differ in their ability to fix N_2, soybean requires approximately 390 kg N ha^{-1} to produce 5000 kg ha^{-1} of with soybean acquiring approximately 50-60% of N through N_2 fixation processes (Salvagiotti et al., 2008).

The works of (Herridge et al., 2008b) reviewed existing literature to estimate %Ndfa and employed methods of conversion to estimate total crop N fixed by soybean in the four top soybean producing countries. Those methods have been utilised to create the same estimations based upon FAO statistical data for 2023 (Table 1.3, FAOSTAT, 2023). Therefore, estimates of total crop N fixed by soybean amount to 16.4 Mt across the top four soybean producing countries, with 13.7, 7.7, 2.2 and 1.1Mt being fixed in Brazil, United States (U.S.), Argentina and China, respectively. Despite the ability of soybean to fix N, large amounts of N fertiliser are still applied (Table 1.4.), hence improving the efficiency of N_2 fixation processes has potential to reduce fertiliser application having economic and environmental benefits.

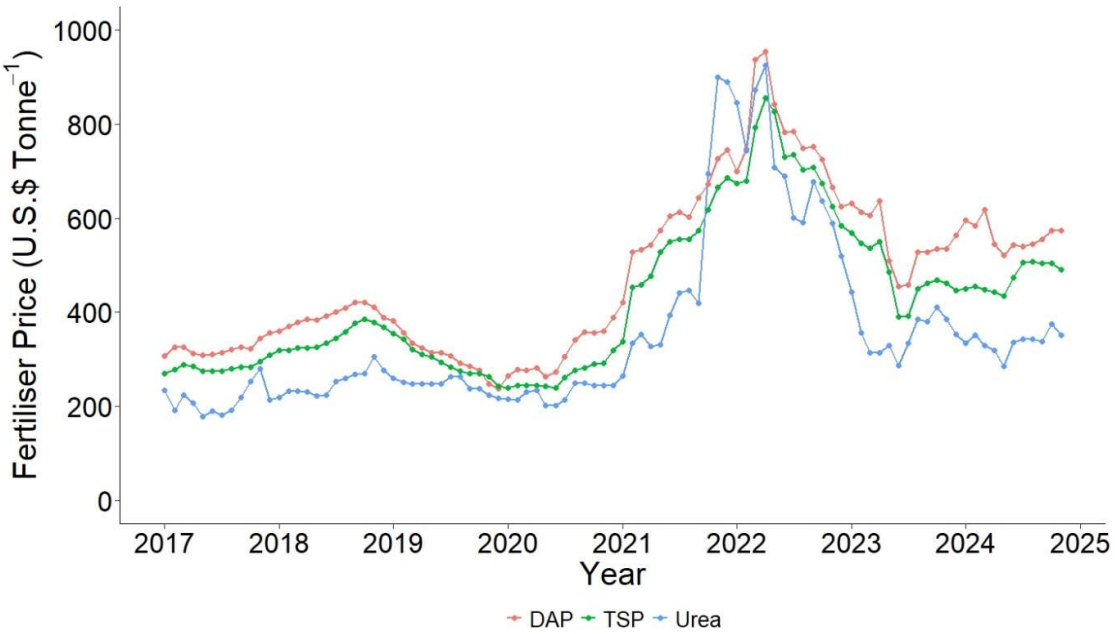


Figure 1.1. Global fertiliser price for diammonium phosphate (DAP, red), triple superphosphate (TSP, green), and Urea (blue) from January 2017 to November 2024. Adapted from World Bank (2024).

Table 1.2. Estimation of N_2 fixed by soybean annually in the four major soybean-producing countries, using FAO statistical data for 2023 (FAOSTAT) and adapted methods from Herridge et al. (2008).

Country	Production (Mt)	Shoot DM (Mt)[a]	Shoot N (Mt)[b]	Crop N (Mt)[c]	% Ndfa	Crop N fixed (Mt)
Brazil	152.1	380.3	11.4	17.1	80	13.7
U.S.	113.3	283.3	8.5	12.8	60	7.7
Argentina	25.0	62.5	1.9	2.8	60[d]	2.2
China	19.5	48.8	1.5	2.2	50	1.1
Total	**309.9**	**774.9**	**23.3**	**34.9**	**68**[e]	**16.4**

[a]Using harvest index value of 0.4
[b]Using %N shoots of 3%
[c]Multiplying shoot N by 1.5
[d]Collino et al., 2015
[e]Average %Ndfa

Table 1.3. Mineral fertiliser use fixed by soybean annually (2018 in Argentina, Brazil and China, and 2023 in U.S.) in the four major soybean-producing countries, adapted from Luddermann (2022). Nitrogen fertiliser use was not reported for Argentina.

Country	Phosphorus total application (kt P_2O_5 year^{-1})	Phosphorus rate (kg P_2O_5 ha^{-1})	Nitrogen total application (kt year^{-1})	Nitrogen rate (kg ha^{-1})
Brazil	3315.3	92	562.0	16
U.S.	926.1	62.8	243.6	22.4
Argentina	171.9	10	-	-
China	413.1	49	408.0	49

The process of N_2 fixation turns atmospheric N_2 into plant available NH_3, with legume crops being the main source of natural agricultural N_2 fixation. Legume crops form symbiotic relationships with soil bacteria, Rhizobia, where N_2 fixation occurs in exchange for carbon (C). The nitrogenase enzyme that catalyses N_2 fixation, requires approximately 52% of nodule respiratory energy (Rainbird et al., 1984). This requires high rates of respiration to produce sufficient Adenosine triphosphate (ATP) and an adequate supply of oxygen (O_2), with the reduction of N_2 requiring 16 ATPs (Kahn et al., 1998).

The N_2 derived from fixation (Ndfa) of soybean differs considerably dependent on environment and management practices amongst countries of production (Herridge et al., 2008b; Santachiara et al., 2019). The inhibitory effect of soil N on N_2 fixation is widely recognised, with N_2 application reducing nitrogenase activity, increasing O_2 diffusion resistance inside the bacteriod and triggering complex hormone signalling to control regulatory mechanisms (Eaglesham et al., 1983; Stougaard, 2000; Streeter, 1985; Vessey and Waterer, 1992). Soil water stress (including deficit and saturation), and non-optimal temperatures, pH, radiation, CO_2 concentration, and nutrient availability have all been reported to have inhibitory effects on N_2 fixation (Divito and Sadras, 2014; Hungria et al., 2000; Minchin and Pate, 1975; Prudent et al., 2015; Serraj and Sinclair, 1996).

1.1.3. Soybean nodules

Soybean nodules, specialised root organs allowing the host of rhizobia to fix N_2, are of the determinate type characterised by the lack of persistent nodule meristem and spherical shape (Hirsch, 1992). Determinate nodules usually export reduced nitrogen as ureides (allantoin and allantoic acid) compared to amides (asparagine and glutamine) exported in indeterminate nodules (Day et al., 2001; Karr et al., 1990; Troitskaya et al., 2000). Nodules remain on the root for a few weeks, then nodule senescence is trigged through accumulation of nitric oxide and nodules are replaced by new ones (Hichri et al., 2016).

Adequate O_2 supply is essential to regulating nodule activity, with low nodule concentration limiting respiration and thus ATP production and N_2 fixation whereas high concentration has risk of denaturing the nitrogenase enzyme (Dong et al., 2001; Marchal and Vanderleyden, 2000). Optimal O_2 fluxes are buffered in the nodule through the presence of four major and minor leghaemoglobins, with nodule leghaemoglobin concentration being closely associated with N_2 fixation efficiency in nodules (Dakora, 1995; Sato et al., 2001).

The formation of nodules is driven by two primary processes leading to nodule morphogenesis and the formation of infection sites (Downie, 2014). The autoregulation of nodulation (AON) regulates N_2 fixation through adjusting nodule number and suppressing rhizobial infection events following systemic long-range root and shoot signalling processes (Nishida and Suzaki, 2018). Various environmental conditions activate critical components of the AON pathway to inhibit additional formation (Isidra-Arellano et al., 2020; Li et al., 2022b). Nodule characteristics such as larger nodule size have been shown to be associated with greater fixation capacity, with more nodules providing more sites for active N_2 fixation (Tajima et al., 2007; Voisin et al., 2003).

1.2. Phosphorus fertiliser

1.2.1. Agronomic advantages of phosphorus fertiliser

Phosphorus (P) is an essential nutrient for plant growth and development and has played a significant role in agricultural production since the 20th century. Phosphorus is a key element in photosynthesis, respiration, biosynthesis of nucleic acids and membranes, and enzyme regulation (Hawkesford et al., 2023; Xu et al., 2019). Phosphorus exists within plants as organic phosphate esters or inorganic phosphate, with tissue inorganic P separated into the metabolically active pool located in the cytoplasm and cellular stored in the vacuole and the main pools of organic phosphate esters being nucleic acids, phospholipids, phosphorylated metabolites and proteins (Veneklaas et al., 2012).

Phosphorus has limited bioavailability in soil with low proportions (typically less than 10%) of total P readily available for plant uptake (Darch et al., 2014). As a result, P must be applied to soils to minimise deficiency and maintain crop yield, with approximately 60% of P applied to cropland coming from phosphate rocks and the remainder coming from recycled P in organic residues (Cooper et al., 2011; Liu et al., 2008). This inefficient uptake of P by crops provides many opportunities for innovation and improvement at the plant-soil interface (George et al., 2017). It has been estimated that improved P utilisation by plants or more efficient P application could negate the need for arable land expansion of approximately 500 Mha to meet crop demand by 2050 (Mogollón et al., 2021).

1.2.2. Negative costs of phosphorus fertiliser

The non-renewable resource of P rock reserves are unevenly distributed globally, with more than 75% of global reserves being found in Morocco, making the resource geopolitically unstable (Cordell and White, 2015). Alongside this P fertiliser is inaccessible to approximately a sixth of the world's farmers due to high and often volatile prices (Figure 1.1) and ineffective P governance (Cordell and White, 2014; Haygarth and Rufino, 2021). Agriculture is considered to be a major cause of eutrophication in many catchments globally, with the one-way flow of P from mineral reserves to farms, freshwaters and into oceans considered beyond the safe operating space for human development (Carpenter et al., 2011; Elser and Bennett, 2011; Withers et al., 2014). Eutrophication is one of the most common causes of poor water quality status of inland and marine waters, disrupting aquatic ecosystems and having detrimental consequences on ecosystem goods and services, human health and economies (Bennett et al., 2001; Moal et al., 2019; Smith et al., 1999). Estimated costs of remediating effects of eutrophication in the U.S. have been calculated as approximately $2.4 billion annually (Wurtsbaugh et al., 2019).

1.3. Role of phosphorus in soybean production

Recent research has argued that alleviating soil P deficit could produce sufficient food without expanding crop area to 2050 (Mogollón et al., 2021); however, this still requires P fertiliser additions of approximately 1000000 Kt (McDowell et al., 2024; Sattari et al., 2012). The wide geographical area of soybean production, with the four top soybean producers spanning three continents, leads to unique challenges in managing P use because of the large variability

in soils, growing seasons and climate coupled with varying access to P fertiliser resources and management practices. Through the manipulation of global topsoil plant available P datasets (McDowell et al., 2023), it is estimated 50% of soils in the top four soybean producing countries are P limited for soybean production, less than or equal to 9 mg P kg^{-1} (Figure 1.2). Argentina and Brazil are estimated to be predominantly P limited (91.3 and 97.1% of soils) whereas this is much lower for China and the U.S. (15.5 and 59.2% of soils). In contrast, 18% of soils in the top four soybean producing countries had excess soil available P, greater than 20 mg P kg^{-1} (0.4, 0.2, 20.5 and 27.8% for Argentina, Brazil, China and the U.S., respectively).

Soybean requires approximately 25 kg P_2O_5 to yield 1000kg of seed (Bagale, 2021). Phosphorus is required for the multistep processes of nodule development, maintaining functional nodule tissue and the provision of ATP (Jemo et al., 2010; Li et al., 2011; Santachiara et al., 2019). However, the underlying mechanisms of soybean response, particularly the processes involved in N_2 fixation, are still poorly known (Xu et al., 2024). Critical soil P test levels for soybean production have been estimated to be approximately 9 mg P kg^{-1} Olsen's extractable P, with soil P maintenance levels required between 9 and 20 mg P kg^{-1} (Culman et al., 2020; Ferguson et al., 1987). A range of P fertiliser recommendations for soybean exist given the large global distribution, but all follow the principle of applying P fertiliser if soil P test value is below the critical level (Fixen and Grove, 1990). Example phosphorus fertiliser recommendations have been summarised in Table 1.4.

Table 1.4. Phosphorus (P) fertiliser recommendations for soybean with differing yields, adapted from Gerwing and Geldermann (2005).

Yield (kg ha^{-1})	**Soil test phosphorus (Olsen's extractable P, mg P kg^{-1})**				
	< 3	3 – 6	6 – 9	9 – 20	> 20
	Fertiliser recommendations (kg P_2O_5 ha^{-1})				
2017	44.8	25.8	11.2	0	0
2690	60.5	34.7	11.2	0	0
3362	75.1	43.7	12.3	0	0
4035	89.7	52.7	14.6	0	0
4707	105.4	61.6	16.8	0	0
5380	119.9	69.5	20.2	0	0

Robust recommendations and guidance for soybean specific P fertiliser use, including actual and optimal rates is challenging and often relies on limited or outdated experimental work (Lyons et al., 2023; Rotundo et al., 2022). Traditionally, fertiliser recommendations were derived from dedicated fertiliser trials, however recent advances in soybean breeding coupled with limited resources has limited the ability to conduct such trials on these newer varieties (Bender et al., 2015; Rotundo et al., 2022). Alongside this current fertiliser recommendations and critical thresholds for soybean are often derived from localised research (for example within states in the U.S.), whilst this is helpful for allowing soil and climate specific recommendations for that area, such drastic differences in fertility philosophies, interpretations and recommendations is challenging for employing guidelines on a more global or larger scale outside of the immediate area of focus (Lyons et al., 2023;

Zhang et al., 2021). For example, soils in tropical regions, such as the Brazilian Cerrado, account for large proportions of soybean production however the soils are highly weathered (low available P) or P fixing soils (added P fertiliser becomes bound to iron and aluminium oxides making it less available) and so fertiliser recommendations derived from experimental work in these regions would not be relevant to regions commonly cultivated within the U.S. (Riskin et al., 2013).

Large proportions of soils within the top four soybean producing countries have been estimated to have soil available P concentrations below the critical thresholds for soybean production (Table 1.4, Figure 1.2). As a result, it is suggested that increasing P fertiliser addition could aid in the closure of soybean yield gaps to meet production demands. However, whilst the obvious solution to this is to increase P fertiliser application, it must be noted that other climatic, soil and management conditions have not been considered, and a blanket increased addition poses risk to the environment as well as significant economic costs. To ensure the balance between improving soybean yield and closing yield gaps a clear understanding of soybean response to P fertiliser addition (including N_2 fixation, plant partitioning, P behaviour and yield parameters) is required to inform the sustainable use of P fertiliser.

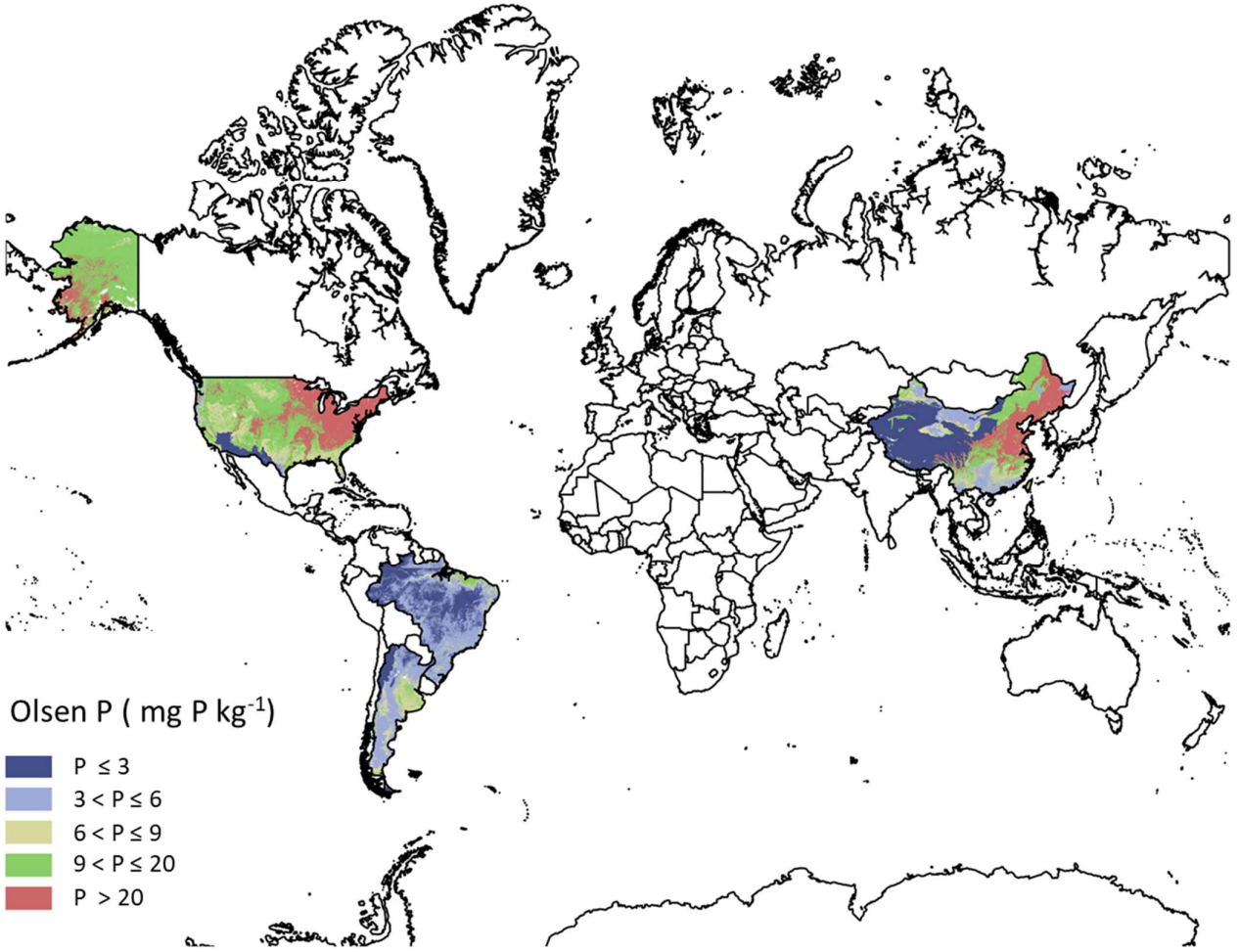


Figure 1.2. Topsoil Olsen phosphorus (P) concentration (mg kg^{-1}) for the top four soybean producers (Argentina, Brazil, China and the U.S.) at a 1 km^2 resolution, adapted from (McDowell et al., 2023). Colours are determined based upon soil P test values with corresponding fertiliser recommendations (Table 1.4.).

1.4. Thesis aims and hypotheses

It is suggested that soybean crop growth is often actually P limited and improving the efficiency of P fertiliser use could contribute to the closure of existing yield gaps to meet the increasing demand for soybean production. As a result, it is **hypothesised that P fertiliser addition can optimise N_2 fixation processes and increase yield.** As a result, this thesis aimed to answer the following questions:

1. How does P fertiliser addition improve nitrogen fixation?
2. What is driving the increase in soybean yield following P fertiliser addition?
3. Does the relationship between P fertiliser addition and soybean yield differ at the global scale?

A conceptual diagram summarising the aims and questions of this thesis can be seen in Figure 1.3. In doing this, this thesis contributes to the body of work on P fertiliser use in soybean production and will help build a greater understanding of the effects of P fertiliser addition to inform future fertiliser guidelines, crop models, and management practices to aid in achieving sustainable soybean production through improved yield and optimised P fertiliser use.

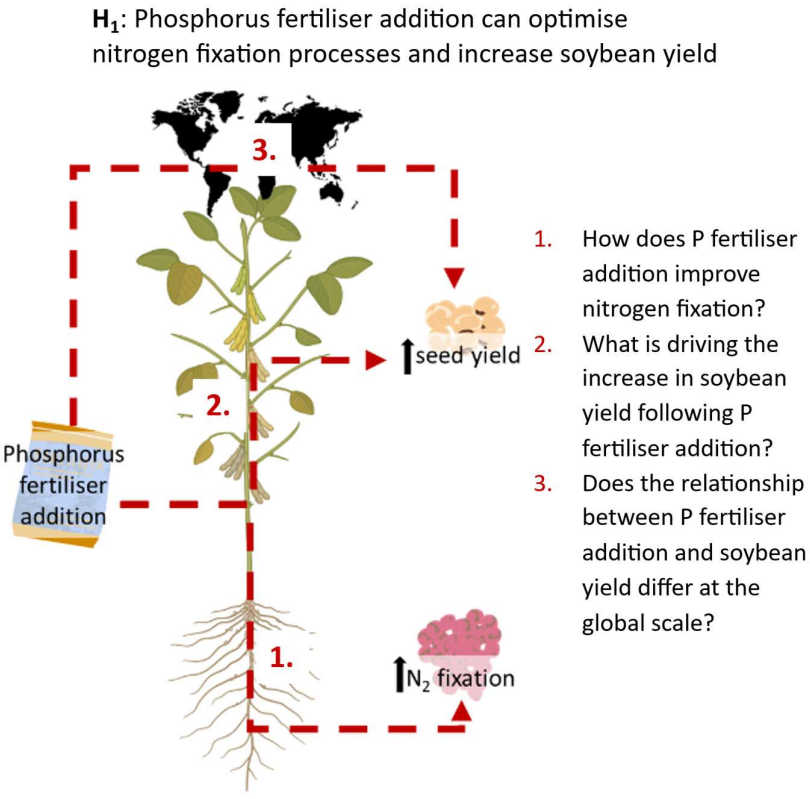


Figure 1.3. Conceptual diagram outlining key questions addressed in this thesis.

1.5. Thesis structure

This thesis consists of four chapters intended for publication and concludes with a general discussion chapter. Chapters four and five comprise of data derived from one controlled environment study. Preliminary experimental work used to inform research completed in chapters four and five is presented in Appendix B. Full details of the thesis structure and associated aims and hypotheses of chapters is summarised below:

Chapter two addresses question three through a meta-analysis of current research on the response of soybean to P fertiliser addition. To improve the understanding of P addition in global soybean production, and the influence of environmental and management factors on the relationship between P addition and seed yield, the specific objectives were to:

1. Quantify the effect and magnitude of P fertiliser on soybean plant growth and yield, with the hypothesis that *P fertiliser addition increases soybean plant growth and yield through improved N_2 fixation processes.*
2. Investigate the variability of the relationship is dependent on explanatory variables, with the hypothesis that *the relationship between P fertiliser addition and yield would be influenced by P fertiliser type, rate of application, and soil biogeochemical properties.*

Chapter two is currently in review in Field Crops Research.

Chapter three aims to evaluate the relative abundance of ureide (RAU) method of determining N_2 fixation of early maturity genotypes under two differing experimental settings.

Chapter four addresses question one through a controlled environment study to examine how P fertiliser addition improved soybean N_2 fixation across the growth cycle, with the hypothesis that there are two, potentially interacting pathways, in which P fertiliser addition will lead to enhanced N_2 fixation:

1. Phosphorus fertiliser addition will drive an increase in nodule formation through increasing resource accumulation and allocation
2. Phosphorus fertiliser addition will drive an increase in nodule function through increased rhizobia efficiency

Chapter four also addresses question two through coupling N_2 fixation analysis with the combined analysis of photosynthetic capacity, morphological traits and yield parameters to better understand how P fertiliser addition increases soybean seed yield.
Chapter four is intended for publication.

Chapter five addresses question two through a controlled environment study to examine how within plant P remobilisation differed between varieties and P fertiliser treatment, and the implication on soybean yield parameters. The chapter aims to address the following hypotheses:

1. Phosphorus fertiliser addition will improve soybean yield parameters through increased uptake and remobilisation.

2. Varieties will differ in yield response to P fertiliser addition because of differences in P uptake and remobilisation strategies.

Chapter five is intended for publication.

Chapter six provides a general discussion and presents the wider findings from across the thesis, with suggestions for future research, and will accept and reject the hypotheses set in this chapter.

2. Influence of environmental and management factors on the effectiveness of phosphorus fertiliser to improve soybean yield: a global meta-analysis

In this chapter, current research on the response of soybean to P fertiliser addition has been studied to improve the understanding of P fertiliser addition on soybean yield, and the influence of environment and management factors on this relationship, using a meta-analysis approach.

2.1. Introduction

Soybean (*Glycine max* L. (Merr.)) is a globally important crop, in terms of both total production and international trade, and the largest source of vegetable oil and animal protein feed (Balboa et al., 2018; Riskin et al., 2013; Vogel et al., 2021). Currently, the global production of soybean is 339 million tons, with 90% of crop production occurring in only five countries (Brazil, United States, Argentina, China and India). Whilst soybean production levels have rapidly increased in the last few years, further increases are required to meet demand (Weiner, 2017). One avenue to increase global soybean production is through closing yield gaps to increase production on existing cropland (Edreira et al., 2017). Soybean yield gaps under rainfed conditions in Brazil, Argentina and the United States have been estimated to be 22, 42 and 32%, respectively (Di Mauro et al., 2018; Merlos et al., 2015; Sentelhas et al., 2015). Altering management practices provides an opportunity to close these gaps, with improving crop nutrition being suggested as an effective strategy (Balboa et al., 2018; Edreira et al., 2017).

To optimise crop nutrition, plant nutrient requirements and soil nutrient supplies are as important. Soybean is a legume that can biologically fix atmospheric nitrogen (N_2), thus reducing the requirements for N fertilisers (Rotaru and Sinclair, 2009). For other macronutrients, such as phosphorus (P) and potassium (K), soybean relies on the uptake of soil available fractions and additional nutrients supplied. Soybean requires approximately 25 kg of P_2O_5 to yield 1000 kg of seed (Bagale, 2021). Mean P fertiliser rate within the top five soybean producing countries was 41 kg P_2O_5 ha^{-1} in 2022 (Ludemann et al., 2022), with an estimated 10% of total P fertiliser used in soybean (Bagale, 2021; Sattari et al., 2014). Current fertiliser-use strategies seek to build soil P levels to meet crop requirements and maximise yield response to P applications dependent on soil types and management practices (Rotundo et al., 2022).

The capacity of soybean to biologically fix atmospheric nitrogen (N_2) reduces the reliance of the crop on synthetic nitrogen fertilisers, with an average of 50-60% of soybean N demand being met by biological N_2 fixation (Salvagiotti et al., 2008). This process requires a large amount of P, as N_2 fixing legumes require more P than legumes utilising only mineral N (Hernandez et al., 2009). Whilst the understanding of the specific mechanisms is limited, it is widely recognised that P must be supplied at adequate levels to optimise the complex process of nodule formation and development (Li et al., 2018). Following nodulation, low availability

of P in the soil limits the biological N_2 fixation processes because P is required to maintain functional nodule tissue (Zhu et al., 2020). Previous meta-analysis estimated the effects of environment on soybean production and underlying yield-determining physiological processes (e.g., (Pierson et al., 2018; Rotundo and Westgate, 2009; Santachiara et al., 2019). However, they have not studied the effect of P fertiliser on soybean production in isolation or in the detail that is required for improved P management.

This study aimed to improve the understanding of P effects in global soybean cropping systems, and the influence of environmental and management factors on the relationship between P addition and seed yield. A meta-analysis approach was used, and the specific objectives were to:

1. Quantify the effect and magnitude of P fertiliser on soybean plant growth and yield
2. Investigate the variability of these relationships dependent on explanatory variables.

It is hypothesised that P fertiliser addition increases soybean plant growth and yield through improved N_2 fixation processes. This relationship would be influenced by P fertiliser type, rate of application, and soil biogeochemical properties. Phosphorus fertiliser addition has been measured and explored in many studies globally; however, to the best of our knowledge, this is the first meta-analysis that evaluates the effect of P fertiliser addition on specific soybean plant growth parameters.

2.2. Methods

2.2.1. Data compilation

The scope of the literature search was confined to studies that controlled for and quantified P fertiliser application rates and reported at least one measure of soybean growth or yield determining parameters. Suitable greenhouse, hydroponic and field experiments published in peer-reviewed journals were collated through a systematic review of the electronic search engines Web of Science (https://www.webofscience.com/) and Scopus (https://www.scopus.com/), completed between November 2021 and November 2023. Based on the aims and objectives of this study, the following search query was established: (ALL=("soybean" OR "glycine max" OR "soyabean" OR “soya” OR “soy”)) AND (ALL=("P" OR "organic P" OR "inorganic P" OR "mineral P" OR "fertiliser P" OR "fertilizer P" OR “P fertiliser" OR "P fertilizer" OR "superphosphate" OR "phosphate" OR "phosphorus" OR “phosphorous”)) AND (ALL=("yield" OR "biomass" OR "growth rate" OR "harvest" OR "dry matter" OR "BNF" OR "N_2 fixation " OR “nitrogen fixation” OR "nodulation" OR "nodule")).

Search results were then organised; authors and journal titles were hidden to reduce researcher bias on inclusion/exclusion. Only peer-reviewed publications of original studies were included, and all repetitions were removed. Efforts were made to find or translate publications into English, but if no translations were found all non-English publications were removed. Well-recorded experimental design (experimental designs with replication, which included reporting of sample number and means at a minimum), reported data on P fertiliser application rates and control treatments, and plant response variables (measure of biomass, seed yield, nodulation or N_2 fixation) were required for a study to be included. Where data

generated from the same study was reported in multiple publications, only the most recent publication was included, these were identified by matching authors, experimental set-up and resulting data. Studies undertaken greater than 25 years ago were excluded as were studies lacking P fertiliser treatment or soybean as a study crop. Only those studies that quantified rates of P fertiliser application and applied a P fertiliser in isolation, rather than a compound fertiliser, were included to enable the isolation of P driven response and enable the quantification of rate of P fertiliser addition. Full details of the screening process are presented in Figure 2.1. From 2520 publications 74 studies qualified as appropriate for inclusion in the meta-analysis (Appendix A Table 8.1.).

Data were extracted on certain plant response variables and relevant explanatory variables, designed to fit the objectives of the study (Table 2.1). When data were only available as a figure, we used free digitising software for extraction WebPlotDigitiser (https://apps.automeris.io/wpd/). Explanatory variables were categorised based on a combination of known soybean crop growth recommendations and agronomic assumptions from the wider literature to best represent the data. Fertiliser application rates were categorised as ≤ 40 kg P ha^{-1} or > 40 kg P ha^{-1} which were lower or higher than the average fertiliser use in the top five soybean producers (Ludemann et al., 2022). Conversions were made of all necessary reported units of measurement for matching representation. Soil available P concentrations were standardised to Olsen P using the following equations: Olsen P = 1.376 Truog P + 0.06, Olsen P = 0.49 Bray-1 P + 3.1, Olsen P = 0.47 Mehlich-3 P + 2.4 (Mallarino & Atia, 2005; McDowell et al., 2021; McDowell et al., 2023). Phosphorus fertiliser addition was standardised to kg P ha^{-1} employing reported depth and bulk density, or a standard of 25 cm depth and 1.2 g cm^{-3} bulk density if not reported, and a conversion of P = 0.436*P_2O_5.

Table 2.1. Response and explanatory variables added to the fixed-effects model described in section 2.2 of the methods. Due to response variables being calculated based on relative response of control vs phosphorus fertiliser treatment, these become unitless.

Response variables (unitless)	Explanatory variables (and category definitions)
Seed yield	Fertiliser application rate; ≤ 40 kg P ha^{-1}, > 40 kg P ha^{-1}
Pod number	Fertiliser type; single superphosphate, triple superphosphate, rock phosphate
Shoot dry weight	Soil pH; acidic (<6.5), neutral (6.5 – 7.5), alkaline (>7.5)
Plant dry weight	Soil available P (Olsen extractable phosphorus): ≤10 mg P kg^{-1}, > 10 mg P kg^{-1}
Root dry weight	Continent of study
Nodule number	Climate of study
Nodule dry weight	Experiment type
Rate of nitrogen fixation	

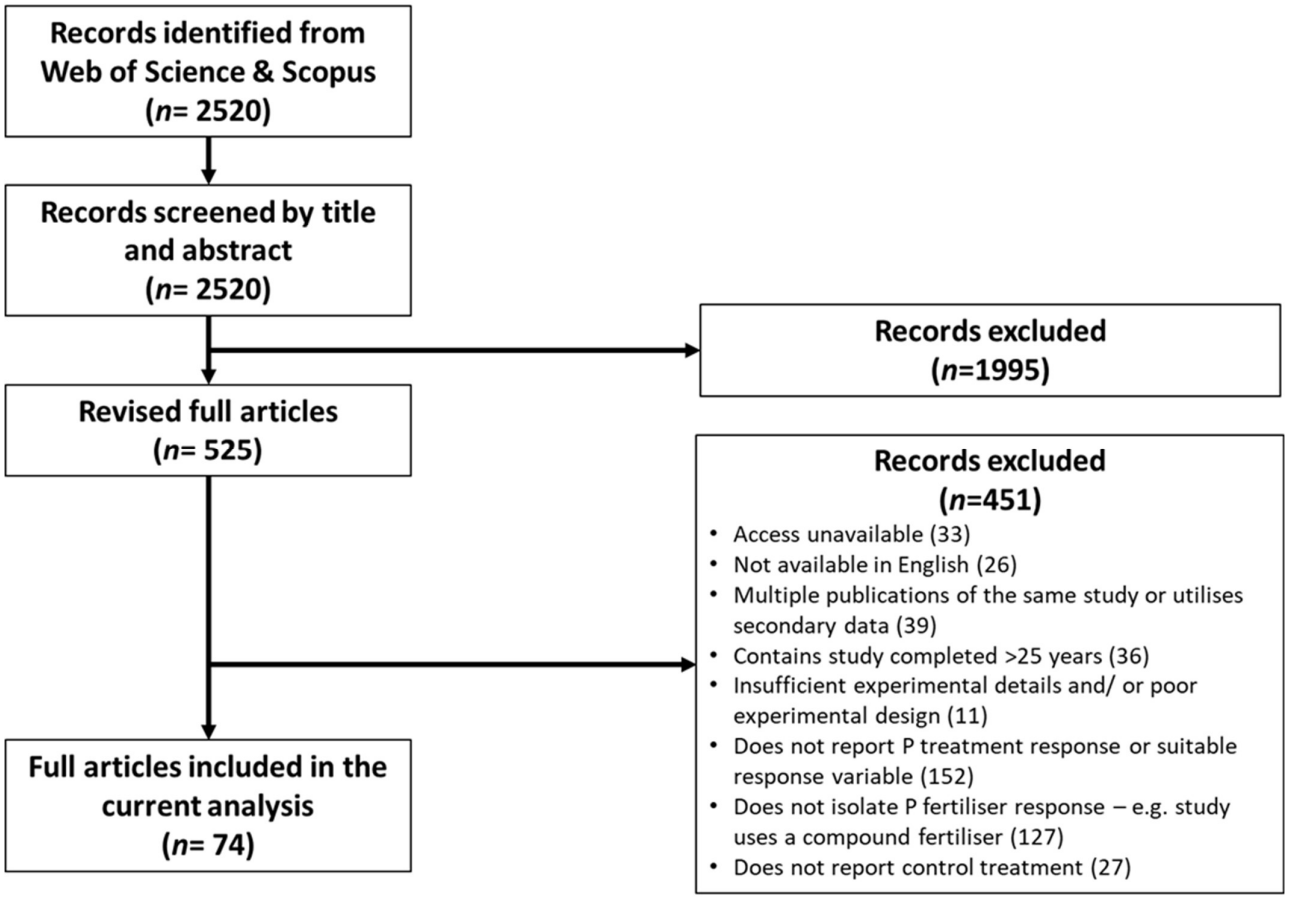


Figure 2.1. Detailed process of inclusion and exclusion criteria used to select scientific articles for this meta-analysis.

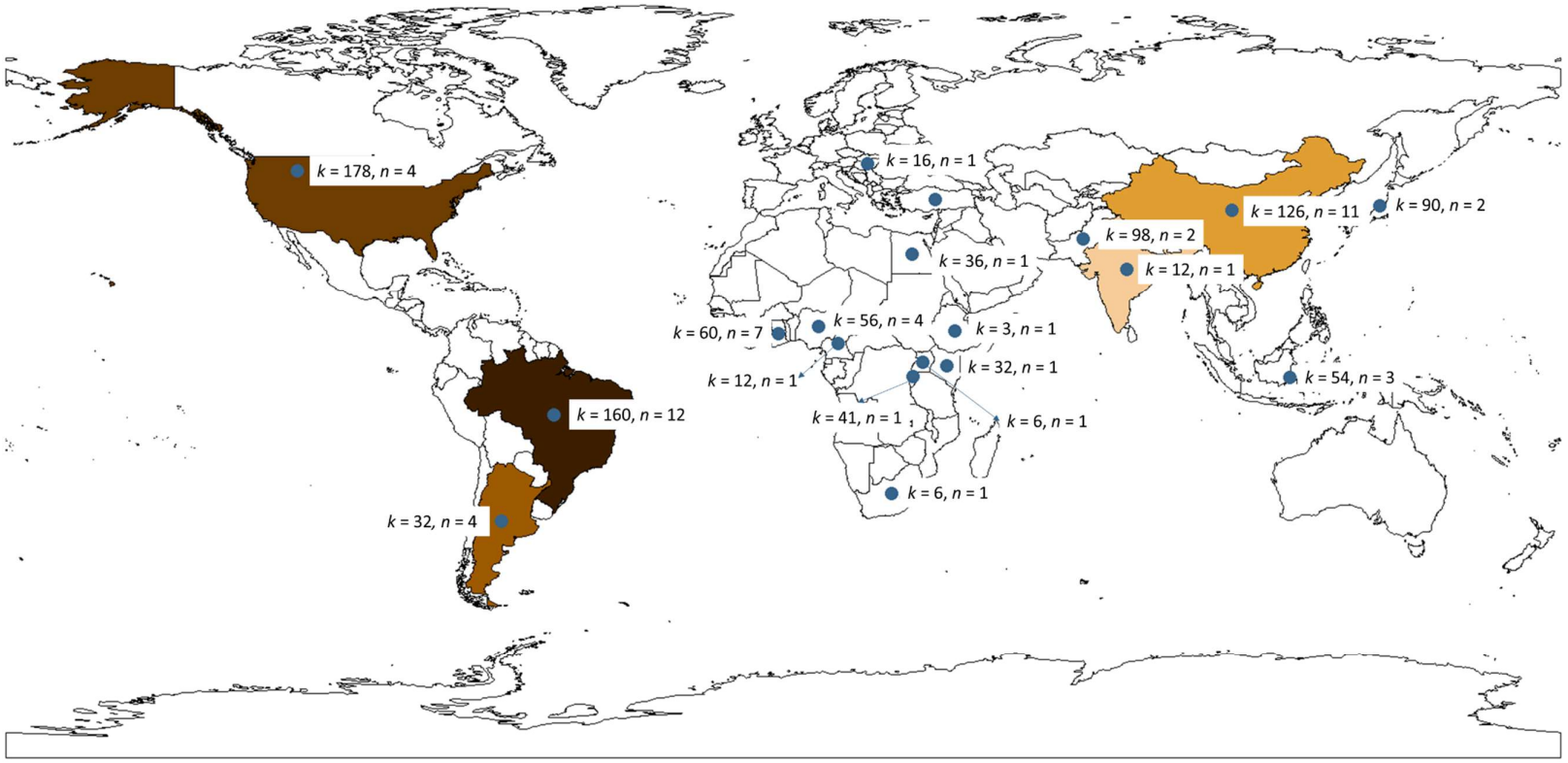


Figure 2.2. Geographic distribution of cases (*k*) and studies (n) included in the meta-analysis and the major global soybean producers. The top five soybean producers (Brazil, United States, Argentina, China and India) are ranked from dark to light according to 2021 production levels (FAO, 2021). The blue dot represents the location of observations included in the analysis, with the number of cases (*k*) and number of publications (n) shown next to the location. Studies and cases that did not report location have not been included in this visualisation.

2.2.2. Statistical analysis

Response ratios were calculated using a control and a treatment response from the included studies, the control response was defined as 0 kg P ha^{-1} fertiliser addition. Data from different seasons, locations and/or experimental situations within each article were considered independent observations. We calculated 1169 effect sizes (*k*) with the package 'METAFOR' from RStudio (version 4.3.1.), using control and treatment means, standard deviation (SD) of plant response, and sample size (*n*). The 'escalc (measure = "ROM")' function was used to calculate the log transformed ratio of means (*lr*) allowing for responses to be weighted according to *n* and SD (Viechtbauer, 2010). As a standard rule, *lr* = 0.2, *lr* = 0.4 and *lr* = 0.8 represent a small, medium and large response (Trepel et al., 2024). When SD was not reported two methods were used to calculate it. First, when only standard error (SE) was reported, SD was calculated using the following function: SD=SE x sqrt (*n*); second, when both SE and SD were not available the average coefficient of variation (CV) across responses reporting SE or SD were calculated, the missing SD was then approximated by multiplying the reported mean by the average CV (Mezeli et al., 2020; Van Groenigen et al., 2011). Publication bias was assessed in the whole dataset, and the sub-set data using funnel plots and Egger's regression test.

The overall model estimated mean *lr* across all plant response variables and experimental types was calculated. A fixed-effects model employing the 'rma.mv' function was used for fitting all data using the default weighted least squares (Viechtbauer, 2010). Evidence of underlying heterogeneity within the dataset was testing using Q statistics, where the Q *p*-value is significant, there is unexplained variability in the dataset, suggesting the need for the use of explanatory variables within the model. Experiment type (field, greenhouse or hydroponic) were added to the model as an explanatory variable, with *lr* for each category being assessed for significant P treatment response, through comparing to zero, and significant differences between category responses. Experiment type categories were also tested for the effect they had on the between response heterogeneity (QE statistic) and the power to explain a significant proportion of the variability (QM statistic). Plant response variables (pod number, seed yield, plant dry weight, shoot dry weight, root dry weight, nodule number, nodule dry weight and rate of N_2 fixation) was fitted as explanatory variables in both the hydroponic systems only and field and greenhouse studies. Statistical analysis on plant response variables and Q statistics were completed as above.

For all further analyses, studies completed in hydroponic systems were removed. These studies comprised of a small number *lr* (125/1170), had a mean *lr* (0.591) significantly greater than the greenhouse and field experiments ($p < 0.001$) and due to the nature of the systems, did not contain moderator variables relevant to the aims and hypotheses of the study. Due to the significant ($p < 0.05$) response observed in different plant response variables, the data was then subset with further analysis only completed on seed yield *lr*. Seed yield was selected due to comprising of the largest *k* and being deemed most relevant to the aims and hypotheses of this study.

Explanatory variables were added to the base model and tested for their effect on the heterogeneity response and explanatory power of the observed seed yield response using

QE, QM and pseudo R^2 statistics. These explanatory variables included year of study, country of study, experimental system, addition of inoculant, P fertiliser type, P fertiliser application rate, soil available P, soil pH and soil texture. They were selected based upon relevance to aims and hypotheses to the study and included when they were reported in more than five studies. Explanatory variables were further tested by performing analysis of variance (ANOVA)s to compare the full model to one excluding the explanatory variable being tested until only those explanatory variables having a significant *p* value remained (Supplementary A Table 8.2).

To explore the potential mechanisms of P fertiliser addition and soil properties on the response of seed yield, selected explanatory variables (P fertiliser type, P fertiliser application rate, soil available P, soil pH, continent of study and climate of study) were examined and the significance of P addition response within each category compared to zero, and significance between explanatory response categories was tested. Explanatory variables were tested for the effect on the between response heterogeneity (QE statistic), ability to explain significant proportion of the variability (QM statistic) and the proportion of variance explained by the explanatory variable (pseudo R^2). Pseudo R^2 was calculated using the function: R^2=(QT−QE)/QT, where QT is the total heterogeneity and QE is the residual heterogeneity after accounting for the explanatory variable. These explanatory variables were selected based on the percentage of reported cases, and those that were deemed to fit the hypotheses and aims of this study. The definitions of categories assigned to these explanatory variables were determined based on the wider literature and common scientific understanding (Table 2.1). When a study did not report the explanatory variable, the cases were not included in the analyses and displayed in the figures.

2.3. Results

2.3.1. Effect of phosphorus addition on soybean productivity

Across all plant response and experiment types, P fertiliser addition had a positive impact on soybean productivity, with an overall model estimated mean *lr* = 0.421 ± 0.004 (Figure 2.3). All plant response types *lr* with experiment type were included as a moderator in the model. Mean effect sizes for all experiment types had significantly ($p < 0.001$) positive response to P addition, with hydroponic experiments having the greatest *lr*, 0.591, greater than the overall mean. With experiment type included in the model QE was high and QM was significant, showing significant heterogeneity that is not explained by experiment type alone (Table 2.2). A large Q statistic shows high underlying heterogeneity within the model (Q = 12839, df = 986, $p < 0.001$). Egger's regression test indicates significant publication bias towards P fertiliser addition having a positive effect ($p < 0.0001$), however this may also be a factor of the high levels of heterogeneity within the data, with the funnel plot shown in Appendix A Figure 8.1.

Figure 2.4 summarises *lr* with plant response type included as an explanatory variable in the model. For studies in greenhouse and in the field, the overall mean *lr* = 0.388 (Figure2. 4.a.). All plant responses were significantly different from zero ($p < 0.001$), except for rate of N_2 fixation ($p = 0.180$). Pod number and nodule number both had significantly greater ($p<0.05$) *lr* compared to other plant responses (*lr* = 0.633 and *lr* =0.591, respectively). For studies

conducted only in hydroponic systems, the mean *lr* = 0.591 (Figure 4.b.). All plant responses were significant different from zero ($p < 0.001$) except for plant dry weight (p =0.149). Seed yield had the greatest *lr* (*lr* = 1.76) followed by nodule dry weight (*lr* = 1.18). In field and greenhouse studies, our analysis revealed that plant response type explained approximately 72% of the variance in effect sizes (pseudo R^2 = 0.72), however the high QE statistic implies that while plant response type is an important factor other sources of variability are also present.

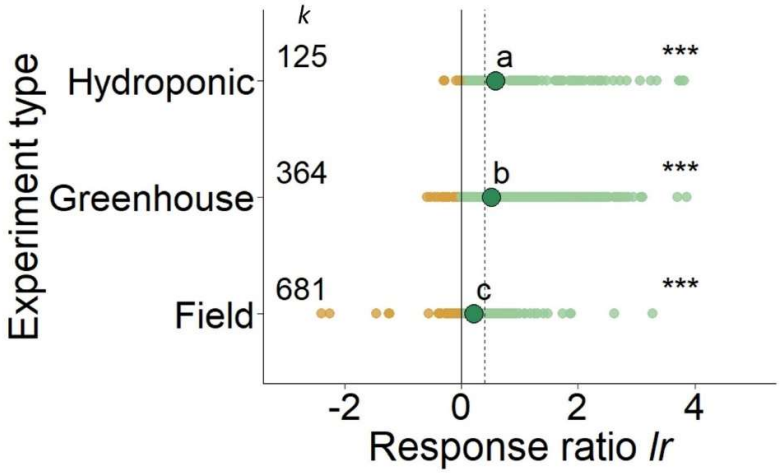


Figure 2.3. Response ratio (*lr*) of soybean response variables (pod number, seed yield, plant dry weight, shoot dry weight, root dry weight, nodule number, nodule dry weight and rate of nitrogen fixation) to phosphorus fertiliser addition. Results are grouped according to experiment type. The dashed line is the overall mean across all cases (*k*), the solid line shows no response. The large dot represents the mean response ratio *lr* for each category, with letters representing significance at the 5% level between categories. Each point in the background indicating an individual case (a pairwise comparison of phosphorus fertiliser addition vs control in a study), showing the distribution of the data. *** denotes a significant phosphorus response at the 1% level when compared to control. Green points represent a positive effect, yellow points represent a negative effect, and grey points represent a non-significant effect. The number of cases (*k*) for each grouped mean *lr* can be seen on the left side of the figure.

Table 2.2. Q statistic results of addition of explanatory variables added to separate models. Explanatory variables were tested for the reduction they had on between-response-heterogeneity (QE statistic) and the ability to explain significant proportion of the variability (QM statistic). Results are displayed as Q statistic, *p*-value and degrees of freedom.

Explanatory variable	QE statistic (between response heterogeneity)	QM statistic (ability to explain significant proportion of the variability)	Pseudo R^2 (proportion of explained variance)
Experiment type	10940.86, df = 957, $p < 0.0001$	4465.46, df = 3, $p < 0.0001$	0.338
Plant response type (field and greenhouse studies	19135.25, df = 1037, $p < 0.001$	8466.88, df = 8, $p < 0.001$	0.724
Plant response type (hydroponic studies)	932.74, df = 118, $p < 0.001$	2561.36, df = 7, $p < 0.001$	0.302
Phosphorus fertiliser type (seed yield *lr* in field and greenhouse studies)	3786.72, df = 455, $p < 0.001$	632.29, df = 4, $p < 0.001$	0.260
Phosphorus fertiliser rate (seed yield *lr* in field and greenhouse studies)	3260.91, df = 456, $p < 0.001$	1158.11, df = 3, $p < 0.001$	0.140
Soil pH (seed yield *lr* in field and greenhouse studies)	1420.72, df = 455, $p<0.001$	1595.49, df = 4, $p < 0.001$	0.357
Soil available phosphorus (seed yield *lr* in field and greenhouse studies)	37341.32, df = 456, $p <0.001$	687.70, df = 3, $p < 0.001$	0.153
Continent of study (seed yield *lr* in field studies)	13896.86, df = 454, $p <0.001$	Q1629.35, df = 5, $p < 0.001$	0.561
Climate of study (seed yield *lr* in field studies)	648.26, df = 402, $p <0.001$	711.93 df = 5, $p < 0.001$	0.521

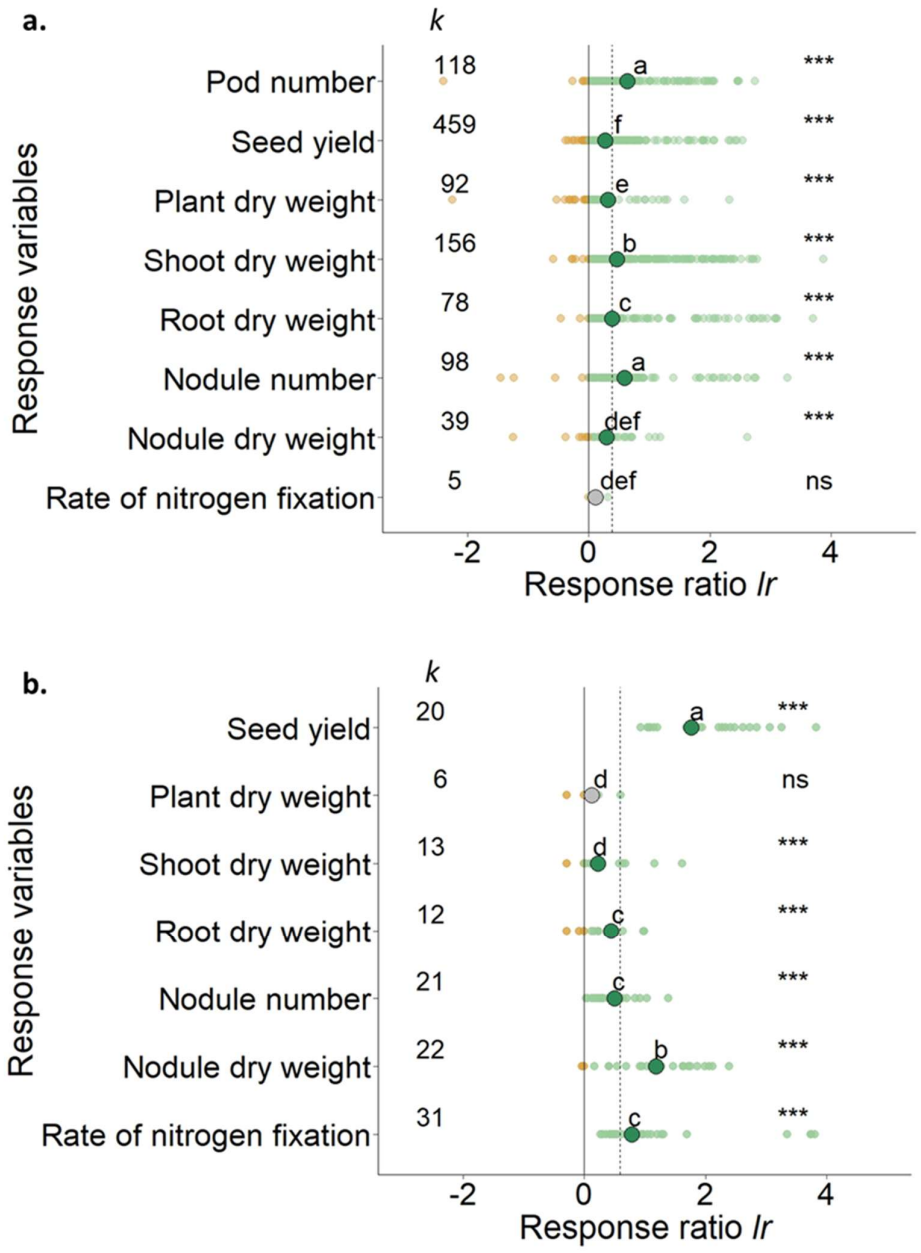


Figure 2.4. Response ratio (*lr*) of soybean productivity across all selected plant response variables to phosphorus fertiliser addition. Figure **2.4.a.** Soybean plant response variables under field and greenhouse experiment systems, **2.4.b.** Soybean plant response variables under hydroponic systems. The dashed line is the overall mean across all cases (*k*), the solid line shows no response. The large dot represents the mean response ratio *lr* for each category, with letters representing significance at the 5% level between categories. Each point in the background indicates an individual case (a pairwise comparison of phosphorus fertiliser addition vs control in a study), showing the distribution of the data. *** denotes a significant phosphorus response at the 1% level when compared to control. Green points represent a positive effect, yellow points represent a negative effect, and grey points represent a non-significant effect. The number of cases (*k*) for each grouped mean *lr* can be seen on the left side of the figure.

2.3.2. Effect of explanatory variables on soybean yield

Following the reduction in cases to examine only seed yield response, and after removing the hydroponic studies, the overall model estimates a *lr* of 0.209 ± 0.009, df = 458. A large Q statistic was observed in the seed yield model, Q = 3830.99, highlighting large amounts of underlying heterogeneity in the dataset. The Egger regression test indicates significant (p < 0.001) publication bias towards P fertiliser addition having a positive effect (funnel plot shown in Supplementary Figure 1). When examining the explanatory variables in isolation, all had high QE statistics and significant QM statistics, showing these variables alone do not explain underlying heterogeneity in the dataset (Table 2.2). When all explanatory variables deemed relevant and reported across multiple studies were fitted to the main model, a significant QE (p < 0.001) was observed, meaning there is still evidence of underlying heterogeneity in the data that cannot be explained by all the explanatory variables combined. When considering the results of the ANOVAs comparing full model and model without year of study, country of study, experimental system, fertiliser rate, fertiliser type and soil pH all had a significant (p < 0.001) p-value, suggesting these variables significantly affected the underlying variability observed in seed yield *lr* to P addition. The pseudo R^2 = 0.741, suggesting approximately 74% of the variance was explained by these variables, however, because the QE p-value remained significant, this observation is not significant because there is evidence of underlying heterogeneity in the data that cannot be explained by the combination of explanatory variables. As a result, selected explanatory variables deemed relevant to the study are discussed further, however due to significant QE statistic, these explanatory variables do not fully explain the response observed in seed yield *lr* following P addition.

Different P fertiliser types (single superphosphate, triple superphosphate and phosphate rock) had significantly (p < 0.05) different seed yield *lr* (Figure 2.5.a). All P fertiliser types had a significantly positive *lr*, P addition of all fertiliser types increased seed yield *lr*. Single superphosphate had a significantly (p < 0.05) greater response followed by triple superphosphate and rock phosphate (*lr* = 0.287, *lr* = 0.191, *lr* = 0.141 respectively). Rock phosphate was used in the lowest number of cases (k = 56) compared to single and triple superphosphate (k = 118 and k = 273, respectively). Whilst both P fertiliser application rates had significantly (p < 0.05) positive seed yield *lr*, application rates > 40 kg P ha^{-1} having a significantly (p < 0.05) greater response than rates < 40 kg P ha^{-1}, *lr* = 0.376 compared to *lr* = 0.193 (Figure 2.5.b). Across both soil available P categories, a significantly (p < 0.05) positive seed yield *lr* was observed (Figure 2.5.c). Soils with low available phosphorus (Olsen P) ≤ 10 mg P kg^{-1} had a significantly (p < 0.05) greater response than Olsen P > 10 mg P kg^{-1} (*lr* = 0.203 compared with *lr* = 0.155). Soil pH differed significantly (p < 0.05) in seed yield *lr* (Figure 2.5.d). Acidic and alkaline soils had a significantly (p < 0.001*)* greater seed yield *lr* (0.352 and 0.369 respectively) compared to neutral soils (*lr* = 0.088).

When considering the P addition response under only field conditions, experiments in all continents had a significantly (p < 0.001) positive seed yield *lr* (Figure 2.5.e). Continent of study explained approximately 56%, with climate of study explaining approximately 52% of the variability in seed yield response to P addition in field experiments (Table 2.2). South America had the greatest *lr* (0.379), followed by Asia and Africa, with North America having significantly lower *lr* (0.054). Across all climate types, under field conditions, a significantly (p

< 0.001) positive seed yield *lr* was observed (Figure 2.5.f). Hot desert and semi-arid climates had a significantly greater (p <0.05) seed yield *lr* (0.413 and 0.310, respectively) than Subtropical-humid and Tropical climates (0.289 and 0.269, respectively), with Continental climates having the significantly lowest (p < 0.05) seed yield *lr*.

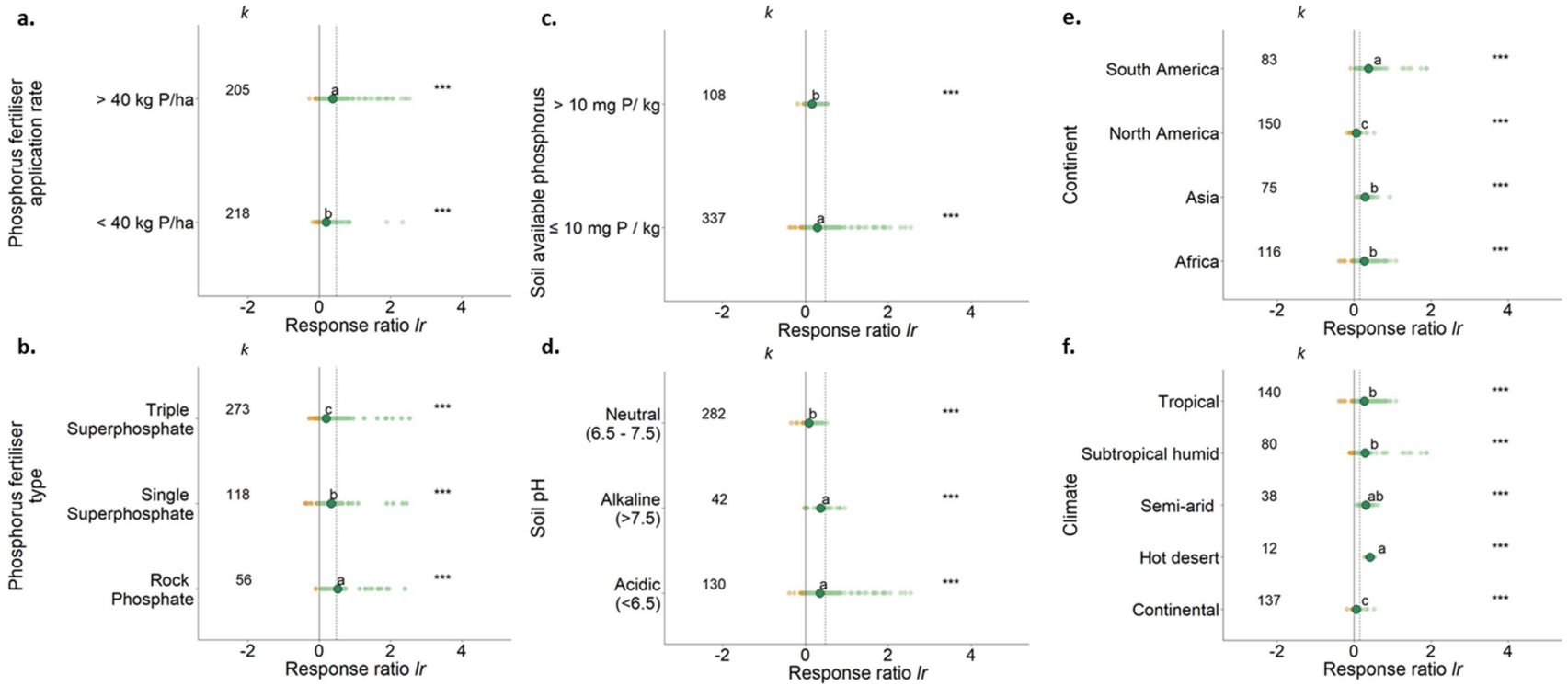


Figure 2.5. Response ratio (*lr*) of seed yield response to phosphorus fertiliser addition, with explanatory variables fitted. **Figure 2.5.a.** Seed yield in field and greenhouse studies with phosphorus fertiliser rate (40 kg P/ha is the average fertiliser use in the top five soybean producing countries) fitted, **2.5.b.** Seed yield in field and greenhouse studies with fertiliser type fitted, **2.5.c.** seed yield in field and greenhouse studies with Olsen extractable soil available phosphorus fitted, **2.5.d.** seed yield in field and greenhouse studies with soil pH fitted, **2.5.e.** soybean seed yield in field studies with continent of study fitted, **2.5.f.** soybean seed yield in field studies with climate of study. The dashed line is the overall mean across all cases (*k*), the solid line shows no response. The large dot represents the mean response ratio *lr* for each category, with letters representing significance at the 5% level between categories. Each point in the background indicating an individual case (a pairwise comparison of phosphorus fertiliser addition vs control in a study), showing the distribution of the data. *** denotes a significant phosphorus response at the 1% level when compared to control. Green points represent a positive effect, yellow points represent a negative effect, and grey points represent a non-significant effect. The number of cases (*k*) for each grouped mean *lr* can be seen on the left side of the figure.

2.4. Discussion

2.4.1. Plant responses to phosphorus addition

Pod number had a significantly greater response to P addition (from cases reported under field and greenhouse conditions) than other plant response variables. The evidence indicates that P addition does not have an effect on seed filling, and that the effect on yield is defined by increasing pod numbers early in the development cycle (Chen et al., 2021; Kerkhoff et al., 2005; Santachiara et al., 2019; Tekulu et al., 2020). Early season P supply is critical for optimum crop yield, with P promoting healthy root system and P uptake and storage for reallocation at later development stages, through translocation of inorganic phosphate via the use of root phosphate transporters (Grant et al., 2001; Lambers, 2022; López-Arredondo et al., 2014). Early season P supply leading to increased pod formation, is likely explained by increased photosynthetic rates following P addition. Three biochemical processes control leaf photosynthesis rates: rubisco activity, electron transport rates and triose-P utilisation; under P sufficient conditions and optimal P uptake, leaf photosynthesis is not limited by ATP availability or rubisco activity (Kaschuk et al., 2009; Sharkey, 1985). Sub-optimal supplies of soil phosphorus can reduce photosynthesis through negative effects on vegetative crop growth of leaf area development, and the photosynthetic capability of the leaf (Taliman et al., 2019).

It was hypothesised that P fertiliser addition increases soybean plant growth and yield through improved N_2 fixation processes. The limited reporting (seven studies, of which only two were in field and greenhouse experiments) of N_2 fixation made this effect difficult to quantifying the field and greenhouse studies. A non-significant (p = 0.180) was observed, N_2 fixation was not significantly increased by P addition under field and greenhouse experiments. Nodule number and dry weight increased significantly following P addition, and it is known that this can lead to increased rates of N_2 fixation. Various management practices affect the amount of N that can be fixed by a legume, including plant genetics, seeding dates and spacing, water and phosphorus availability, and soil pH. These agronomic measures play a key role in both nodule formation, through activating regulatory mechanisms and providing sufficient sites for rhizobial infection, and through maintaining rhizobial activity for active N_2 fixation (Nguyen et al., 2020; Thilakarathna et al., 2018). Optimal P status, and other agronomic measures promote biomass production required for partitioning of carbohydrates to be translocated to nodules, enabling the survival of the rhizobia (Kopke and Nemecek, 2010). Phosphorus limitations can impact nodule formation processes, confirmed with the significant nodule number *lr* to P fertiliser addition observed, with it being reported that the syntheses of mitochondrial and symbiosome membranes, essential mechanisms for nodule formation, are limited under P limitation (Sulieman and Tran, 2015).

Nitrogen fixation rates did not have a significant *lr* with nodule number having a significantly greater *lr* compared with N_2 fixation and nodule dry weight, suggesting increased nodule number may not lead to an increased N_2 fixation rate. This implies that energy and resources are being utilised in the symbiotic relationship to form more nodules but not maintained to allow for the active functioning and N_2 fixation processes. However, this result may be merely a function of the meta-analysis, if studies that observed nodule dry weight and/or N_2 fixation

occurred in N sufficient soils where there was no demand for N_2 fixation because of direct uptake from soil N supplies. Unfortunately, soil N status could not be used as an explanatory variable because of the lack of consistent reporting across experiments. It is also important to note that only five cases from one study reported rates of N_2 fixation. When looking at N_2 fixation rates and nodulation in hydroponic systems, where k = 31, nodule number and rate of N_2 fixation did not have significantly different *lr*, suggesting P addition increased both nodule number and rate of N_2 fixation. The wider literature does suggest that adequate P levels (achieved through P fertiliser addition) not only leads to an increase in nodule number and biomass, but it also achieves an increase in N_2 fixation rates. Nitrogen fixation requires large energy supplies (ATP) and reductant (NADPH), both of which require large amounts of P (Sa and Israel, 1991); and there is also evidence of P limitation restricting ureide transport from nodules to plants, meaning that fixed N_2 may not be effectively utilised by soybean to produce storage protein, therefore affecting yield and yield quality if P is limiting (Zhu et al., 2020).

2.4.2. Response of seed yield to environmental and management practices

Whilst soybean seed yield *lr* showed a significantly lower response to other plant response types, an approximate 20% increase in seed yield was measured following P addition. If this increase could be upscaled globally it could help to meet the required increase of soybean production. The addition of P fertiliser may also improve seed quality and nutritional status (for example through greater seed protein content) rather than seed yield *per se* (Krueger et al., 2013; Soliman and Farah, 1985). However, seed compositional changes in response to P fertiliser addition are inconsistently reported and could not be assessed in this meta-analysis. We highlight this as a potential area for further research, but one could hypothesise P addition would improve seed quality by increasing protein and oil content. Whilst we assume increased seed yield *lr* following P addition is in response to (at least in part) the increase in nodulation and N_2 fixation *lr* also observed, there are too few observations to confidently quantify this relationship. Seed yield *lr* showed large variability, with the underlying heterogeneity of the data being significant despite the addition of explanatory variables, highlighting the variable nature of the global soybean cropping systems.

Higher rates of P fertiliser addition (categorised as > 40 kg P ha^{-1}) produced significantly greater seed yield *lr*, however, there is much greater variation in this response compared to that from the lower P application category. Whilst this greater *lr* following greater rate of application agrees with fundamental scientific understanding, increasing P addition does not lead to an exponential increase in yields. Critical thresholds are commonly reached and if not properly considered, excessive P fertiliser use can have detrimental impacts on soil and water health, as well as economic disadvantages (Stamm et al., 2021). Soils with soil available P status ≤ 10 mg P/kg had a significantly greater seed yield *lr* than those with greater soil available P status, this is to be expected as estimations of critical P status have been recorded at approximately this level (Sucunza et al., 2018). The relationship between soybean yield *lr* following P addition and soil available phosphorus concentrations is variable, due to several confounding factors, including the availability of other nutrients, soil types and climate and other management practices (McDowell et al., 2024). Single superphosphate and triple superphosphate were much more commonly used in the studies included in this meta-

analysis than rock phosphate; however, rock phosphate had a significantly greater *lr* than these more widely utilised fertilisers, presumably because of the lower number of cases.

Soil pH explained the largest percentage of variance (36%) in seed yield response to P addition, with pH known to have a critical role in P supply. pH, both directly and indirectly influences the solubility of P, determining availability and mobility through biogeochemical processes, such as microbial P cycling and P mineralisation, P adsorption and precipitation capacity of a soil (Marschner et al., 2005). Neutral pH soils had significantly lower seed yield *lr* than acidic and alkaline soils, thought to be because of neutral soils already having greater soil available P status so tend to be less reliance on the addition of P fertiliser. Phosphorus precipitation with calcium is common in alkaline soils, causing it to become rapidly immobilised and in acidic soils P adsorption with aluminium and iron oxides is high (Elbasiouny et al., 2020; Hinsinger, 2001). As a result of the precipitation and adsorption of P at these extremes less P is available for plant uptake, leading to an increase in seed yield response to P fertiliser addition. It must be noted that this increase in response will only be observed if the rate of uptake is greater than rates of precipitation and adsorption, with these rapid processes leading to larger amounts of P fertiliser addition to maintain yield increase in multi-season crop growth.

Continent and climate explain large proportions of the variance in seed yield response to P addition (56 and 53%, respectively), with seed yield *lr* varying significantly between regions and climates. This is a result of several factors, including, but not limited to, soil chemical properties, including soil available P status, soil P retention, climate and other nutrient status, and the large regional imbalances in fertiliser availability. South America, which produces more than half of all global soybean, showed the greatest seed yield *lr* to P addition. The common occurrence of soils with high P binding capacities within South America, such as the deep, highly weathered, high P binding soils of the Mato Grosso region in Brazil, require large rates of P inputs (Riskin et al., 2013; Rotundo et al., 2022; Roy et al., 2017). The greater seed yield *lr* observed in Africa is expected, with generally low P soils within the continent having greater requirements for P fertiliser application; however, this requirement is rarely met in practice with the need for increased fertiliser accessibility to the region being widely recognised, particularly to sub-Saharan Africa (Haygarth and Rufino, 2021; Sanchez, 2015). North America, a continent widely recognised for its high rates of fertiliser use and often soil over-saturated P showed a lower seed yield *lr*, suggesting these cropping systems overall would not benefit from increased P fertiliser application. When considering the influence of P fertiliser addition on seed yield *lr* under differing climates, the general trend observed in the results of this meta-analysis were arid and desert climates showing the greahitest *lr*, followed by tropical and sub-tropical, with continental climates showing the smallest *lr*. Evidence suggests temperature and precipitation can influence soil P cycling and P uptake capacity, with higher evaporation rates and lower rainfall decreasing water availability causing P to become less available (Kang et al., 2012; Wang, 2024). The decreased availability of soil P may lead to increased *lr* when additional P is supplied to the system. However, further work is required to unpick the relationship between climatic variables and soil biogeochemical processes, particularly surrounding P use.

2.4.2. Limitations of study and recommendations for future research

As with many meta-analyses and data synthesis studies, the results presented here, are constrained by several factors. Firstly, the occurrence of publication bias, in both the whole dataset and when looking only at seed yield, suggests that the observed effect sizes may be skewed to favour positive results. This is a common problem with statistically significant results being more likely to be published than studies reporting non-significant results and/or the fact that positive results tend to be published earlier than negative results (Nakagawa et al., 2022). The second, is the statistical power of our findings, particularly the results of explanatory variables are largely limited by the occurrence of these variables being reported in published studies. Whilst we can draw inferences and recommendations from the use of explanatory variable categories, it must be noted that the number of cases that were included in the analysis only represents a small fraction of soybean production. This is resultant from a lack of inclusion of data within scientific reporting, coupled with the unequal distribution of research and production of soybean.

The underlying heterogeneity of the dataset, shown with significant Q statistics, can be associated with the large differences in environmental and management practices resulting from the large global distribution of soybean highlights the need for situational and regional work to better enable P fertiliser recommendation and use to optimise yield. For example, several soil characteristics are known to alter the plant response to P, including soil type, clay content, water level and microbial composition, however the interaction of these characteristics, and the knowledge behind these mechanistic processes is limited in soybean cropping systems. Further work is needed, together with better reporting, to develop a better understanding of soybean specific mechanisms behind P uptake, the role of P in N_2 fixation processes, and its impact on yield. The complex interactions of environmental, management and genotype factors influencing soybean production could lead to disparities in critical soil P threshold levels and P fertiliser recommendations, leading to the mismanagement of P resources. This mismanagement can impact on production, with widescale management practices across farms or regions that do not best optimise the heterogenous nature of the growing systems.

2.5. Conclusions

This analysis aimed to improve the understanding of P fertiliser addition effects in global soybean cropping systems. The results showed an overarching increase in soybean response to P fertiliser addition compared to control treatment, with a mean increase of approximately 40% across all plant response traits, environmental and management types included within this meta-analysis. Whilst N_2 fixation and nodulation was reported in a small number of studies (n=20 combined), these results highlight the notable role P addition has in improving N_2 fixation processes and the critical impact this may have on observed yield responses.

Collectively, the results highlighted the complexity of soybean yield response to P fertiliser addition, indicating that soybean yield cannot be increased by simple management decisions related to increased P fertiliser application. The result is highly dependent on

other management and environmental factors specific to the growing region. Large variability in soybean yield to P fertiliser addition was observed at varying scales, because of several potential drivers: (i) environmental conditions primarily associated with soil biogeochemical conditions (such as soil P status, soil pH, soil water status and soil texture) play a key role in managing and maintaining the availability of P fertiliser applied to cropping systems, (ii) management conditions, including P fertiliser application rates, types and timing of application, influences the availability and uptake of P applied to the crop, and (iii) environmental conditions associated with potential abiotic stressors, such as temperature, rainfall or irrigation practices, and other nutrient availability. To tailor P management strategies to optimise soybean production globally further work needs to understand the role of P in increasing yield under these variable environmental and management conditions, with a focus on the role of N_2 fixation in improving yield.

3. Potential limitations to the quantification of relative abundance of ureides to assess soybean symbiotic nitrogen fixation

The previous chapter, a global meta-analysis of soybean response to phosphorus (P) fertiliser, identified large variations in seed yield response dependent on environment and management conditions, and limited studies investigating soybean nitrogen (N_2) fixation response to P fertiliser addition. In this chapter, the suitability of the relative abundance of ureides for quantifying nitrogen (N_2) fixation was assessed. This chapter is intended for publication as a short communication and has been presented as such. Additional information relevant to this short communication is included in Appendix B.

Dear Editor,

The quantification of relative abundance of ureides (RAU) has been previously calculated alongside techniques for assessing symbiotic nitrogen (N_2) fixation such as $\delta^{15}N$ natural abundance ($\delta^{15}N$ NA), and as such is identified and commonly used as a method for reliable prediction of the N_2 fixation process in certain legume species, including soybean (Herridge et al., 1990; McClure et al., 1980). Current methodologies commonly involve the conversion of RAU to N_2 derived from fixation (Ndfa) based on the equations proposed in the seminal work of Herridge and Peoples (1990). However, recent experimental work has raised questions surrounding the applicability of these equations.

Soybean (*Glycine max* (L.) Merr.) plants have the capacity to fix N_2 from the atmosphere via rhizobia inside root nodules, with Ndfa of soybean differing considerably dependent on environment and management practices amongst countries of production (Herridge et al., 2008b; Santachiara et al., 2019). Reliable estimation of N_2 fixation is essential to guide management decisions to improve soybean N_2 fixation and yield, with N limitation being the primarily limiting resource for soybean yield after water deficit (Giller and Cadisch, 1995; Hirel et al., 2007; Sinclair and Wit, 1975). The RAU method in stem tissue involves the analysis of relative concentrations of forms of nitrogen, described in Equation 1.

$$\boldsymbol{Relative\ Abundance\ of\ Ureide_N}\%(\boldsymbol{RAU}\%) = \left(\frac{\boldsymbol{4U}}{\boldsymbol{4U+N}}\right) \times \boldsymbol{100} \qquad (1)$$

Where U and N are concentrations of ureide and nitrate (Herridge and Peoples, 1990). Nitrogen derived from fixation (Ndfa%) was calculated based on quadratic function proposed by Herridge and Peoples (1990), R^2 = 0.97 (n = 30), between Ndfa and RAU for six genotypes of soybean at five field sites, described in Equation 2.

$$\boldsymbol{RAU}\% = \boldsymbol{1.35 + 0.311 Ndfa}\% + \boldsymbol{0.0057\ Ndfa}\%^{\boldsymbol{2}} \qquad (2)$$

This method can be more favourable as they require less expensive and sophisticated equipment than more traditional isotopic methods (Herridge et al., 1990; Rosso et al., 2021). However, there are a number of variables that can cause potential sources of error through increased levels of stem ureide concentrations inconsistent with rates of N_2 fixation, including abiotic stressors, such as drought, soil N and plant senescence (Aveline et al., 1995; Purcell et al., 2004). Therefore, by analysing soybean N_2 fixation under two environmental and experimental conditions we aimed to evaluate the RAU method of determining N_2 fixation of early maturity European soybean genotypes.

When employing the quadratic and linear functions proposed by Herridge and Peoples (1990) in the controlled environment study, Ndfa was overestimated, with both stem tissue and xylem sap RAU giving Ndfa values greater than 100%, which not only is biologically impossible but almost 200% greater than the Ndfa values recorded using $\delta^{15}N$ NA methods (Table 3.1). In the field trial, where RAU% were much lower, whilst Ndfa values calculated from RAU stem tissue were biologically possible, a large over-estimation was observed, with values being 66.5% greater than those recorded using $\delta^{15}N$ NA methods. When the quadratic function proposed by Herridge and Peoples (1990) was employed to the stem tissue RAU and $\delta^{15}N$ NA data collected in both the field trial and CE study, the large mean squared error (mse = 55.91 and 734.60 respectively) and negative R^2 value (R^2 = -2.77 and -1.87 respectively) suggested the quadratic function does not accurately capture the relationship observed in the datasets (Figure 3.1.a and 3.1.b), the regression equation overestimates Ndfa under the environmental and management conditions of the two studies.

Errors may arise in the estimation of stem RAU because of smaller ureide concentration in the basal section of stem (Patterson and LaRue, 1983; Rosso et al., 2021), and the reliability of petioles for RAU estimation is dependent on the transport of N solutes, with increased ureide accumulation when shoot N demand is greater (Rosso et al., 2021). Nitrogen remobilisation has been found to introduce variability to ureide concentration at earlier growth stages which can impair RAU measurement (Aveline et al., 1995). For example, Purcell et al. (2004) found similar RAU values between nodulating and nonnodulating near isolines of 'Hardee' soybeans. Under water deficit and salinity stress, shoot ureide concentrations have been found to increase despite inhibition of acetylene reduction activity, because of regulatory mechanisms such as decreased rate of ureide catabolism in leaves rather than increased rates of N_2 fixation, leading to the accumulation of ureides in stem tissue out of proportion to N_2 fixation and the overestimation of Ndfa (King and Purcell, 2005; Ladrera et al., 2007; et al., 1999; Serraj and Sinclair, 1996).

The seminal work of Herridge and Peoples (1990) found a trend for the RAU values of early-maturing genotypes to be higher at the same level of $\delta^{15}N$ NA Ndfa. In the 30 years since the regression equations were proposed by Herridge and Peoples, soybean breeding has continued to advance, particularly when considering the focus on early maturity genotypes to meet the demand for increased European soybean production (Rotundo et al., 2024). As a result, the early maturity genotypes used in this study (000 and 0000), and subsequent studies, may also be outliers from the original conversion equation. Poor agreement between with the published relationships between RAU and Ndfa by Herridge and Peoples have also been observed in other experimental studies (e.g. (Purcell et al., 2004; Schweiger et al., 2012).

In conclusion, this work highlights a potential limitation to the RAU method for quantifying N_2 fixation processes, particularly in early maturity genotypes or under stress conditions that may influence ureide metabolism and transport. This limitation should be further tested by examining the correlations between RAU and Ndfa for a range of genotypes and environmental conditions. Whilst we recognise the benefits the RAU method provides, acting as a low-cost alternative to isotopic methods for quantifying Ndfa, there is a need in the scientific community to recognise the potential limitations of the equations proposed for the conversion of RAU to Ndfa, with regular calibration of the techniques required particularly as breeding and management practices continue to advance.

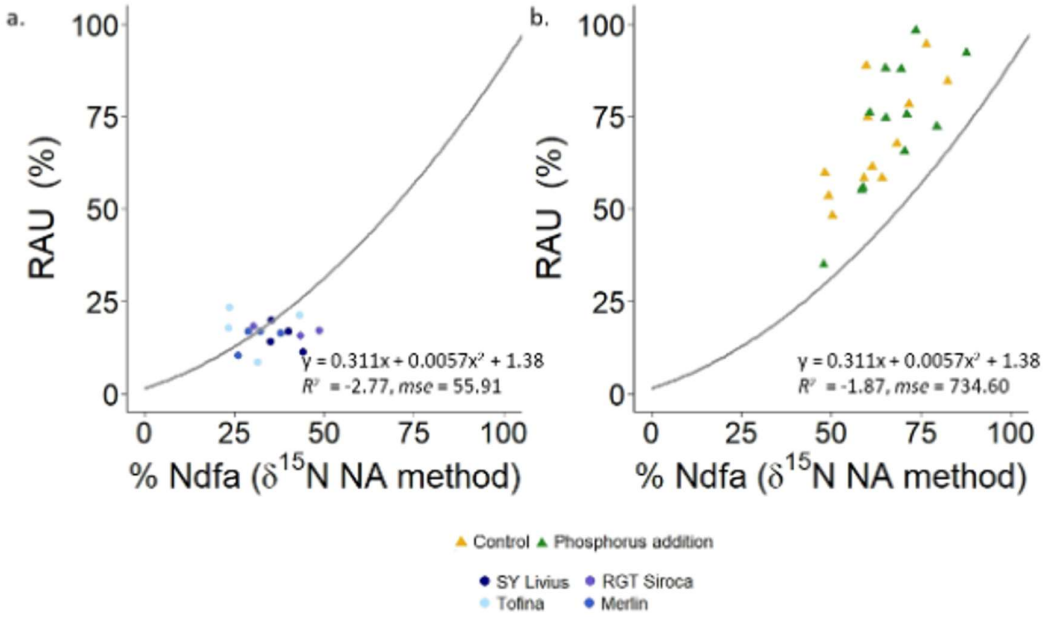


Figure 3.1. Relative abundance of ureides (RAU) measured using relative ureide assay of stem tissue and nitrogen derived from fixation (Ndfa) measured using natural abundance (δ^{15}N NA) methods. **Figure 3.1.a.** Relative abundance of ureides compared to Ndfa at R2 stage of phenological development of four European, early maturity genotypes of soybean grown in a field trial of randomised split plot design with four replicates, where long-term average annual temperature is 9.0 °C and average annual precipitation is 563 mm (Karges et al., 2022). The fitted line is the relationship found by Herridge and Peoples (1990) for vegetative soybean stem tissue ($y = 0.311x + 0.0057x^2 + 1.38$, R^2 – 2.77, mse =55.91). **Figure 3.1.b.** Relative abundance of ureides compared to Ndfa at R1 stage of phenological development of Merlin genotype grown in controlled environment conditions in 0.75 L pots, with 7 replicates and an average day/ night temperature 25/18°C and a 16-hour photoperiod where average canopy PAR was maintained at 800 µMol m^{-2} s^{-1}, under two contrasting phosphorus (P) fertiliser treatments – a control treatment, soil available P concentration of 6 mg P kg^{-1} and no P fertiliser addition, and P addition treatment, soil available P concentration of 6 mg P kg^{-1} and addition of 0.75 g P pot^{-1}, equivalent to 60 kg P ha^{-1}. Colour represents different treatments, with the green and yellow triangles representing P fertiliser addition and control treatments respectively in the controlled environment study, and the different shades of blue circles representing the different varieties in the field trial. The fitted line is the relationship found by Herridge and Peoples (1990) for vegetative soybean stem tissue ($y = 0.311x + 0.0057x^2 + 1.38$, R^2 – -1.87, mse =734.60).

Table 3.1. Nitrogen derived from fixation (Ndfa) and relative abundance of ureide (RAU) measured using relative ureide assay of stem tissue and Ndfa measured using $\delta^{15}N$ natural abundance ($\delta^{15}N$ NA) methods of soybean. Pot trial data represents samples at R1 stage of phenological development of Merlin genotype grown in controlled environment conditions in 0.75 L pots, with 7 replicates and an average day/ night temperature 25/18°C and a 16-hour photoperiod where average canopy PAR was maintained at 800 µMol m^{-2} s^{-1}, under two contrasting phosphorus (P) fertiliser treatments – a control treatment, soil available P concentration of 6 mg P kg^{-1} and no P fertiliser addition, and P addition treatment, soil available P concentration of 6 mg P kg^{-1} and addition of 0.75 g P pot^{-1}, equivalent to 60 kg P ha^{-1}. Field trial data represents samples at R2 stage of phenological development of four European, early maturity genotypes of soybean grown in a field trial of randomised split plot design with four replicates, where long-term average annual temperature is 9.0 °C and average annual precipitation is 563 mm. Data is displayed as mean ± standard error for all treatments One-way ANOVA *p* values are displayed.

Source of Variation		**Stem tissue**		**$\delta^{15}N$ NA**
		RAU (%)	Ndfa (%)	Ndfa (%)
Pot	Control	69.0 ± 4.3	111.4 ± 3.3	62.6 ± 3.1
	Phosphorus addition	73.0 ± 5.2	114.2 ± 4.2	67.3 ± 3.0
	Phosphorus (P)	0.562	0.611	0.288
Field	Merlin	15.1± 1.6	55.7 ± 2.6	31.1 ± 2.5
	RGT Siroca	17.0 ± 0.7	58.8 ± 1.1	40.7 ± 5.5
	SY Livius	15.5 ± 1.9	56.2 ± 2.9	38.5 ± 2.2
	Tofina	17.7 ± 3.3	59.1 ± 5.2	30.3 ± 4.6
	Variety (V)	0.807	0.859	0.187

4. Influence of phosphorus fertiliser on soybean nitrogen fixation and yield

In the previous chapter the suitability of the relative abundance of ureides for quantifying nitrogen (N_2) fixation was assessed. In this chapter, soybean production across the growth cycle following P fertiliser addition has been investigated, focusing on the role of nodule formation and function on N_2 fixation.

4.1. Introduction

Soybean (*Glycine max* (L.) Merr) is a globally important legume, representing 50% of the world legume cropping area and 68 % of legume production (Herridge et al., 2008a). Currently, total global production of soybean is 339 million tonnes, although further increases of approximately 15% are required to meet global demand (Weiner, 2017). Soybean seeds contain approximately 20% oil and 40% protein, with proportionally lower carbohydrate, higher protein, higher poly- and mono-unsaturated fat than other legumes (Messina, 1999). The high demand for nitrogen (N) in soybean production is primarily met through the ability to fix atmospheric N, with an average of 50-60% of soybean N_2 requirements coming from fixation (Salvagiotti et al., 2008). Whilst N_2 fixation processes, achieved through the symbiotic relationship with either natural or commercial inoculant rhizobia, can enhance crop yields by improving N_2 supply and reduce the reliance on synthetic N_2 fertiliser use, the process is energy and resource demanding, with the energy costs estimated to be proportional to that required to produce 25% of shoot dry matter at harvest (Schulze et al., 1999).

Phosphorus (P) is the second most limiting nutrient for crop production (after N), with global yield in approximately 40% of the worlds arable land limited by P availability. Legumes, such as soybean, engaging in symbiotic N_2 fixation have higher requirements for P than non N_2 fixing varieties (Drevon et al., 2015). Two potential mechanisms by which P deficiency impairs N_2 fixation activity include: (1) directly influencing activity due to insufficient resources to meet high rates of energy transfer requirements for the N_2 fixation processes, or (2) indirectly influencing activity through feedback mechanisms occurring when lower shoot growth decreases demand for N_2 fixation (Sinclair and Nogueira, 2018). However, the potential mechanisms for how P deficiency impairs N_2 fixation are not fully understood, with studies of P fertiliser addition on both nodulation and specific N_2 function per unit of nodule mass being limited and often having contradicting effects (Qiao et al., 2007b).

The cumulative benefits of P fertiliser addition for both soybean plant growth and the feedback mechanisms between increased plant growth, plant signalling, and plant-microbial symbiosis to improve N_2 fixation, increases both yield quantity and quality. Whilst P fertiliser addition is known to increase yield in soybean, the pathways and trade-offs of resource exchange and biochemical response at the whole plant level are relatively unknown. Several environmental and management practices are known to influence the yield response following P fertiliser addition (Chapter Two). Improved understanding of the pathways of N_2 fixation response and productivity across the growth cycle are essential to better inform crop management practices to maximise and sustain production.

It was hypothesised that two, potentially interactive, pathways in which optimal P supplies (achieved through P fertiliser application) will lead to enhanced N_2 fixation:

1. Phosphorus fertiliser addition will drive an increase in nodule formation, through increasing photosynthetic capacity and resource accumulation and allocation
2. Phosphorus fertiliser addition will drive an increase in nodule function, through increased rhizobia efficiency and increased rates of fixation.

Through coupling N_2 fixation analysis with the combined analysis of photosynthetic capacity and morphological traits, this chapter aims to better understand pathways and trade-offs to optimise soybean productivity.

4.2. Methods

4.2.2. Experimental design and growing conditions

Two soybean genotypes (Merlin and RGT Siroca – 000 and 00 maturity group, respectively) were grown under two P treatments (control and P fertiliser addition) and harvested at four distinct phases of phenological development (R1 – beginning of flowering, R3 – beginning of pod formation, R5 – beginning of seed formation and R7 – beginning of full maturity). The two varieties used, Merlin and RGT Siroca are two early maturity soybean varieties registered in Europe (000 and 00 maturity groups, respectively), with Merlin being indeterminate growth type registered in 1997 and RGT Siroca being semi-determinate registered in Austrian Federal Office for food Safety (2025). Plants were sown in a randomised complete block design, with 6 pot replicates for each variety, P treatment, and phase of phenological development. Four litre pots were filled with a 3:1 mixture of Leighton Buzzard lime free sand and sandy loam topsoil, to lower soil nutrient status. Characteristics of the sand: soil mix prior to fertiliser treatment are given in Table 4.1 and methods describing soil analysis completed are given in Section 9.2.6. When filling pots, granular magnesium and potassium fertilisers were applied to all pots at rates of 0.15 and 0.4 g pot^{-1}, respectively (18 and 86 kg ha^{-1} equivalent). Phosphorus fertiliser (single superphosphate, 18% P_2O_5) was applied at a rate of 0.75 g P pot^{-1} (equivalent to 60 kg P ha^{-1}) to the P fertiliser addition treatments, with no P fertiliser being applied to the control treatment.

Prior to sowing, seeds were first sterilised by immersion in 70% ethanol for 45 s, rinsed in sterile water, immersed in 5% NaHCl for five minutes, then rinsed in sterile water five times. Following sterilisation, seeds were pre-germinated on sterile filter paper. Following successful germination, seeds were selected for uniformity with two seeds being transplanted at 3 cm depth in the centre of 4 L pots. On transplanting, seeds were inoculated with 1 ml *B. japonicum* USDA110 at a density of 10^8 colony forming units. USDA110 was previously cultured on modified arabinose gluconate (MAG) agar for 7 days at 30 °C. Following successful emergence, at ten days, healthy plants were thinned to one per pot for uniformity. Plants were watered daily to maintain a water holding capacity between 70 - 80%. Plants were grown in a controlled environment room, with an average day/night temperature of 25 °C/18 °C and a 16-hour photoperiod (8:00 - 24:00, with a 30-minute ramping period between 8:00

and 8:30) where average canopy photosynthetically active radiation (PAR) was maintained at 800 $\mu Mol^{-2}\ s^{-1}$. Micronutrients were applied in the form of an adapted N and P free Hoagland's solution (Table 9.3) at a rate of 1 ml l^{-1} of water applied at day ten, thereafter biweekly until harvest.

Table 4.1. Soil characteristics prior to any fertiliser additions calculated from a subsample comprising 10 pseudo-replicates of the mixed sand: soil prior to fertiliser treatment and pot filling.

Pre-treatment soil characteristics	
Sand content (%)	91.2
Plant available phosphorus (mg kg^{-1})	6.1
Plant available ammonium (mg kg^{-1})	1.1
Plant available nitrate (mg kg^{-1})	21.2
pH	7

4.2.3. Photosynthetic performance and dry weight sampling

In the 48 hours prior to harvesting, photosynthetic performance was measured for each plant between the hours of 10:00 & 16:00, in a randomised order, to reduce photoperiod bias. Gas exchange and light-adapted Photosystem II maximum efficiency (PSII efficiency) was measured on the middle leaflet of the most recently fully expanded first trifoliate leaf using an infra-red gas analyser (6400xt LiCor Portable Photosynthesis System, Lincoln, Nebraska, USA) with a fluorescence head. Carbon dioxide was set at ambient levels (390 ppm), radiation at 800 $\mu Mol^{-2}\ s^{-1}$, with a cuvette temperature of 25 °C and ambient humidity (typically 40% to 50%).

At harvest, plants were de-topped below the cotyledons, below-ground material was immediately washed to remove any soil, and nodules were removed and counted by hand. A sub-sample of nodules (0.1 ± 0.01 g) was placed in 1 ml chilled phosphate buffer pH 7 and immediately frozen at -20 °C for leghaemoglobin analysis. Above-ground plant material was partitioned into leaf, stem, pod and seed. It must be noted that at R5, the pod comprises of any seeds present, whereas at the R7, the seed was removed from the pod. Total leaf area was measured using a leaf area meter (Li-3100 Leaf Area Meter, Li-Cor Inc., Lincoln, Nebraska, USA). All partitioned plant tissues were weighed to obtain fresh weight, then dried at 40°C for at least 72 h to obtain dry weight. Specific nodule dry weight was calculated by dividing nodule dry weight by root dry weight and average nodule dry weight was calculated by dividing nodule dry weight by nodule number.

Seed yield was determined at the R7 phase of phenological development following the partitioning of plant dry weight, and the harvest index (HI) was calculated as the ratio of seed dry weight to total shoot dry weight. Pod HI was calculated as the ratio of seed dry weight to total pod and seed dry weight. Number of pods and seed number per pod was determined at

R7. Thousand seed weight was estimated by dividing the seed dry weight by total seed number and multiplying by 1000.

4.2.4. Nitrogen fixation analysis

Relative ureide assay

Dried stem samples of soybean were milled to <1 mm diameter using a ball mill, then extracted in a 0.1 mol L^{-1} phosphate buffer and ethanol heated to 80 °C. Extracts were then cooled, filtered, and centrifuged. Young-Conway's (Young and Conway, 1942) and ninhydrin (Yemm and Cocking, 1955) methods were used to determine ureide and nitrate concentration, respectively, using colorimetry and a spectrophotometer. From this relative abundance of ureide (RAU) was calculated:

$$Relative\ Abundance\ of\ Ureide_N\%(RAU\%) = \left(\frac{4U}{4U+N}\right) \times 100$$

Where U and N are concentrations of ureide and nitrate (Herridge and Peoples, 1990). Full details of RAU methods are given in Section 9.2.3.

Shoot carbon and nitrogen analysis

Shoot tissue was homogenised, based on relative weight within treatment groups, and ball milled to a fine powder. Total N and carbon (C) concentration was determined for 10 ± 1 mg of shoot tissue using an Elemental Vario EL CUBE CN analyser and combined with shoot dry weight were then used to determine total N and C mass.

Leghaemoglobin analysis

The method of (Senthilkumar et al., 2021) was adapted as follows. Approximately 0.1 g of fresh nodules were picked into 1 ml pH 7 chilled phosphate buffer and frozen prior to analysis. An additional 4 ml phosphate buffer was added to the samples that were ground on ice. Samples were then filtered using cheese cloth and centrifuged at 10,000 xg for 20 min at 4 °C. 1 ml of supernatant was further diluted five times, before being added to an equal volume of alkaline pyridine reagent. Sodium dithionite was added to induce a colour change that was measured using a spectrophotometer (Ultrospec 2100 pro, Biochrom Ltd., Cambridge, UK) at 556 nm. Absorbance was compared to a calibration curve comprising known bovine haemoglobin standards between 0 - 100 ppm to calculate concentration, employing tissue weight, volume of phosphate buffer and dilution factor to convert to mg leghaemoglobin g^{-1} nodule.

4.2.5. Statistical analysis

Outliers were determined if greater than two standard deviations from the mean and removed from the dataset. Data were tested for normality using the Shapiro-Wilk test and histograms and Q-Q plots were visually assessed. Where data were not normally distributed, they were transformed using either logarithmic or square root functions to achieve normality.

A three-way analysis of variance (ANOVA) assessed for statistical differences in plant response, with phase of phenological development, P treatment and genotype as factors. To assess pairwise differences following a significant three-way or two-way ANOVA interaction, Bonferroni post hoc tests were applied to control for Type 1 error across multiple comparisons. When only a significant main effect was detected without interactions, Tukey's HSD post hoc test was applied, employing a more powerful approach for multiple comparisons when interactions are not present.

Spearman's rank correlation and significance was completed to explore correlation between nodule traits and N_2 fixation across both P treatments and varieties at all phases of phenological development. Following results of Spearman's rank correlation, the relationships between three key nodule traits and N_2 fixation parameters (leghaemoglobin concentration and RAU, nodule dry weight and shoot N, and nodule number and shoot N) were examined further using analysis of covariance (ANCOVA). ANCOVAs were used to determine if there were differences between nodule traits and nitrogen fixation properties of different treatments (P, variety, and phase of phenological development). Nitrogen fixation parameters were fitted as the main effect, nodule trait as the covariate, and the different treatments as factors. To assess the impact of nodule trait on nitrogen fixation, a linear model was fitted to the data, grouped by factors that had significant interaction with the nodule trait identified by ANCOVAs.

4.3. Results

4.3.1. Nitrogen fixation response to phosphorus addition

A significant ($p < 0.05$) P x phenological development interaction was observed, with Bonferonni post-hoc tests revealing P fertiliser addition significantly ($p < 0.05$) increases RAU at R1 (Figure 4.1.a.). The phosphorus fertiliser addition and control treatments at R3 and R5 phases of phenological development showed no significant difference, with RAU nearing maximum values. No significant difference was observed between P treatments at R7, but R7 RAU was significantly lower than at R3 and R5. Merlin had a significantly higher (p <0.01) RAU at R1 compared to RGT Siroca (mean = 48 and 23%, respectively, whereas at the R7 phase of phenological development RGT Siroca had a significantly higher (p <0.01) RAU compared to Merlin (mean = 58 and 20%, respectively). Both varieties of soybean, exhibited similar patterns in RAU response to P fertiliser addition across the phase of phenological developments, with no significant ($p = 0.630$) P x variety x phenological development three-way interaction observed.

A significant (p <0.01) P x phenological development interaction was observed in absolute shoot N content (g plant^{-1}), with Bonferonni post-hoc tests revealing significant (p <0.01) differences between P treatments at the R1 and R7 phase of phenological development (Figure 4.1.b). Shoot N content was 85.6% higher in P fertiliser addition treatments compared to the control at the R1 phase of phenological development, and 15.6% higher at the R7 growth stage. Shoot N content increased linearly as growth continued, with no significant variety x phenological development interactions ($p = 0.654$), and both varieties followed the same scale of increase in shoot N content. Both varieties of soybean exhibited similar patterns

in shoot N response to P fertiliser addition across the phase of phenological developments, with no significant (p = 0.652) three-way interaction observed (Figure 4.1.b).

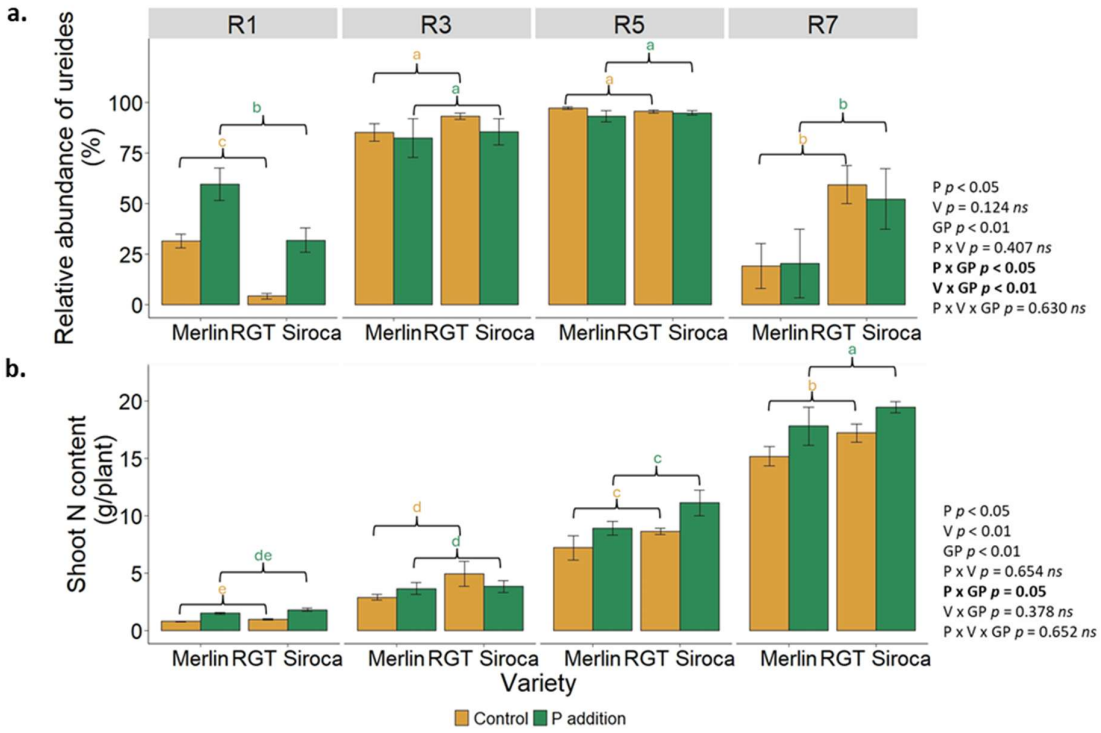


Figure 4.1. Nitrogen (N_2) fixation response of two varieties of soybean (Merlin and RGT Siroca) grown under two phosphorus (P) treatments (control and P fertiliser addition). Individual plants in pots were harvested at four distinct phases of phenological developments (R1, beginning of flowering; R3, beginning of pod formation; R5, beginning of pod filling; and R7, beginning of full maturity). **Figure 4.1.a.** Relative abundance of ureides (%) determined from stem extracts (Herridge and Peoples, 1990) and **Figure 4.1.b.** shoot nitrogen content (g plant^{-1}). Data are means ± SE of 6 replicates. Three-way ANOVA p values are displayed on the right of the figure, with letters of significance above bars indicating results of Bonferroni post hoc tests for phosphorus × variety interactions, where the colour represents the P treatment

4.3.2. Nodule trait response to phosphorus addition

Nodule number per plant had a significant (p < 0.01) P x variety x phenological development interaction (Figure 4.2.a). A Bonferroni post-hoc test revealed a significant (p < 0.05) difference between Merlin P fertiliser addition and control treatments at R7, with P fertiliser addition plants having 77% more nodules than control treatment plants. No other significant differences were observed. Both varieties showed trends of increased nodule number following P fertiliser addition at all phases of phenological developments, except for RGT Siroca at R3. Whilst there was no significant difference between P treatments of the two varieties at the earlier phase of phenological developments, a trend in increased response to P treatment was observed in both varieties at the R1 phase of phenological development, with a 120 and 131% increase in response to P fertiliser addition in Merlin and RGT Siroca, respectively.

The results of Tukeys post-hoc test showed a significantly ($p < 0.01$) greater nodule dry weight following P fertiliser addition compared to the control treatment (Figure 4.2.b). No varietal significant ($p = 0.086$) differences were observed: however, nodule dry weight increased significantly ($p < 0.01$) with increased phase of phenological development. No significant interactions were detected when looking at nodule dry weight. Phosphorus fertiliser addition led to a significant ($p < 0.05$) increase in specific nodule dry weight, nodule dry weight relative to root dry weight (g nodule g root^{-1}), there was an increase in nodule mass relative to root mass (Figure 4.2.c). Phase of phenological development had a significant ($p < 0.01$) effect on specific nodule dry weight, with R1 having the significantly lowest specific nodule dry weight, followed by R3 with R5 and R7 having the greatest specific nodule dry weight. No significant interactions were detected when looking at specific nodule dry weight. Whilst no significant P x phenological development interaction was observed ($p = 0.189$), at R1 there was a trend in increased specific nodule dry weight following P fertiliser addition compared to the control. The average nodule dry weight (mg nodule^{-1}), increased significantly ($p < 0.01$) with phase of phenological development up to R5, and whilst not statistically significant, showed a decreasing trend at R7 (Figure 4.2.d). No significant interaction effects were observed, and average nodule dry weight was not significantly influenced by P ($p = 0.457$) or variety ($p = 0.524$).

A significant ($p <0.05$) P x phenological development interaction was observed in leghaemoglobin concentration (Figure 4.2.e.). Whilst Bonferonni post-hoc tests did not reveal significant differences between P treatments at any phase of phenological developments, there was a visual trend of leghaemoglobin concentration increasing following P fertiliser addition at R1 and R3, with a trend of leghaemoglobin concentration decreasing following P fertiliser addition at R5. Both varieties of soybean exhibited similar patterns in leghaemoglobin concentration response to P fertiliser addition across the measured phases of phenological development, with no significant ($p = 0.805$) three-way interactions being observed. At R7, whilst not statistically significant, the varieties appeared to respond differently to P: Merlin showed no response, while RGT Siroca showed increased leghaemoglobin following P fertiliser addition. A significant variety x phenological development interaction ($p < 0.05$) was observed, with Merlin showing a visible trend in increased leghaemoglobin concentration at the R7.

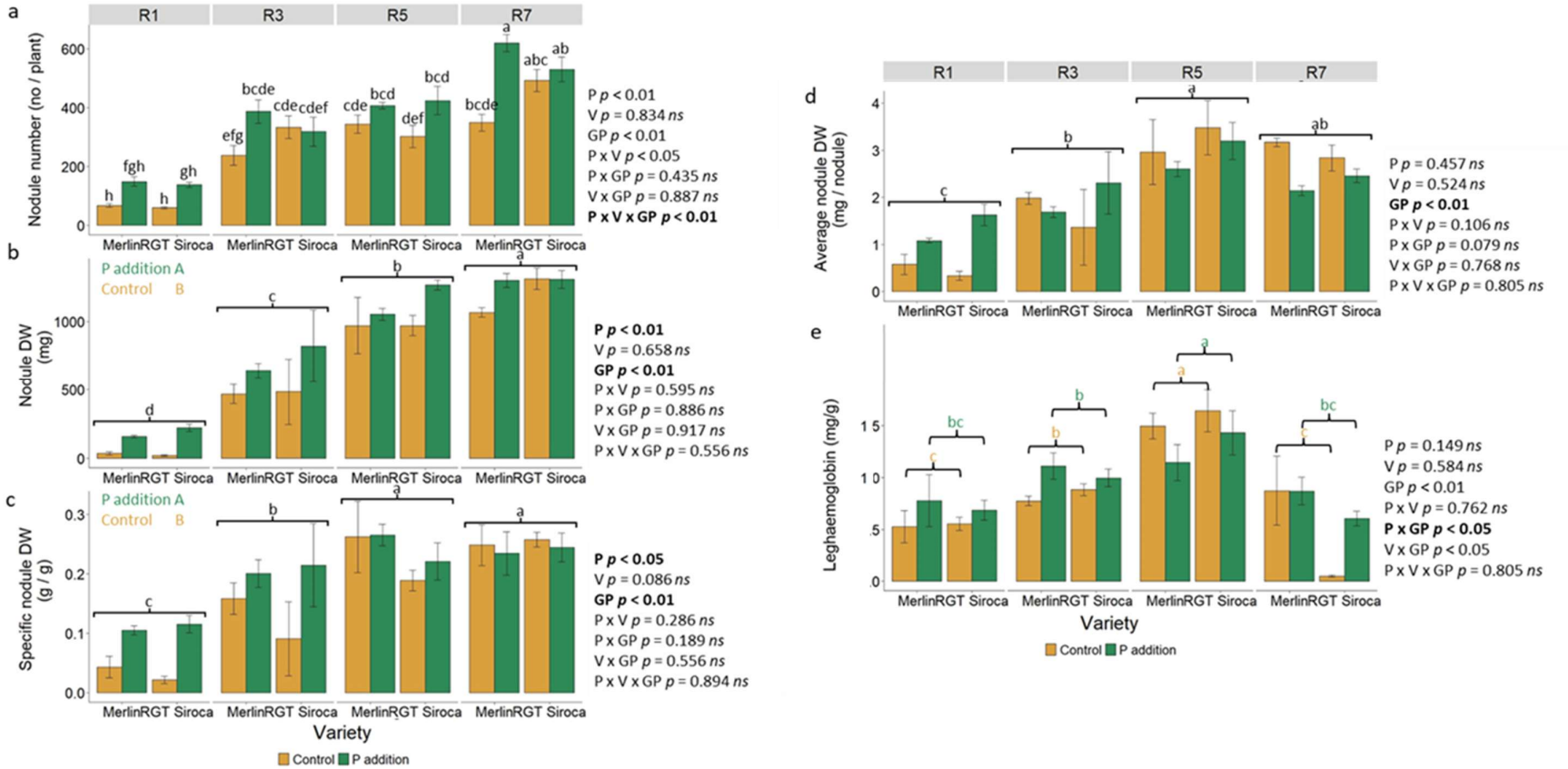


Figure 4.2. Nodulation response of two varieties of soybean (Merlin and RGT Siroca) grown under two phosphorus (P) treatments (control and phosphorus fertiliser addition). Individual plants in pots were harvested at four distinct phases of phenological developments (R1, beginning of flowering; R3, beginning of pod formation; R5, beginning of pod filling; and R7, beginning of full maturity). Data are means ± SE of 6 replicates. Three-way ANOVA *p* values are displayed on the right of the figure, with letters of significance indicating results of post-hoc tests from ANOVA results shown in bold. Colours represent phosphorus treatment. **Figure 4.2.a.** nodule number per plant **Figure 4.2.b.** nodule dry weight (DW, mg) **Figure 4.2.c.** specific nodule DW (nodule dry weight normalised for root dry weight, g g^{-1}) **Figure 4.2.d.** average nodule DW (calculated by nodule DW divided by nodule number, mg $nodule^{-1}$) **Figure 4.2.e.** nodule leghaemoglobin concentration (mg g^{-1}) of a subset of fresh nodules.

4.3.3. Above-ground morphological response and photosynthetic performance response to phosphorus addition

No significant interactions were observed in plant dry weight, however P treatment, variety and phenological development all significantly ($p < 0.05$) influenced plant dry weight. Merlin had significantly ($p < 0.01$) greater plant dry weight compared to RGT Siroca, with P fertiliser addition also having significantly ($p < 0.05$) greater plant dry weight (Figure 4.3.a). Phenological development and P fertiliser treatments were found to be significant ($p < 0.05$), with variety showing no significant effect on leaf area (Figure 4.3.b). Plants under P fertiliser addition had significantly ($p < 0.05$) greater leaf area than those under the control treatments. No significant interactions were apparent for leaf area. A significant ($p < 0.01$) P x phenological development interaction was observed when considering specific leaf area (leaf area per leaf dry weight), with P fertiliser addition having a significantly ($p <0.01$) lower specific leaf area at the R1 phase of phenological development compared to the control, and no significant differences between P treatments at the R3 and R5 phase of phenological development (Figure 4.3.c). A significant ($p < 0.01$) variety x phenological development interaction was observed, with RGT Siroca showing a significantly lower specific leaf area at the R5 phase of phenological development compared to Merlin. No significant three-way interactions were observed.

Net rate of photosynthesis (Anet, µmol m^{-2} s^{-1}) had a significant ($p < 0.01$) interaction between variety x phenological development (Figure 4.3.d). In both the R1 and R3 phase of phenological development, RGT Siroca had a significantly ($p <0.01$) greater net photosynthetic rate than Merlin, whereas at the R5 phase of phenological development there was no significant difference between the varieties. Whilst there was no significant involvement of P within interactions, plants under P fertiliser addition had a significantly greater ($p < 0.01$) rate of photosynthesis than the control plants. No significant three-way interactions were observed. Light-adapted Photosystem II maximum efficiency did not show a significant response to any treatments or their interactions (Figure 4.3.e).

A significant ($p < 0.01$) interaction was observed between P x phenological development in shoot C:N ratio (Figure 4.3.f). At the R1 phase of phenological development, P fertiliser addition displayed a significantly ($p < 0.01$) greater C:N ratio compared to the control, with no P effect being observed at the R3 and R5 phase of phenological developments with C:N ratio remaining constant, while at the R7 phase of phenological development there was a trend in increased C:N ratio under P fertiliser addition. No significant three-way interactions were observed.

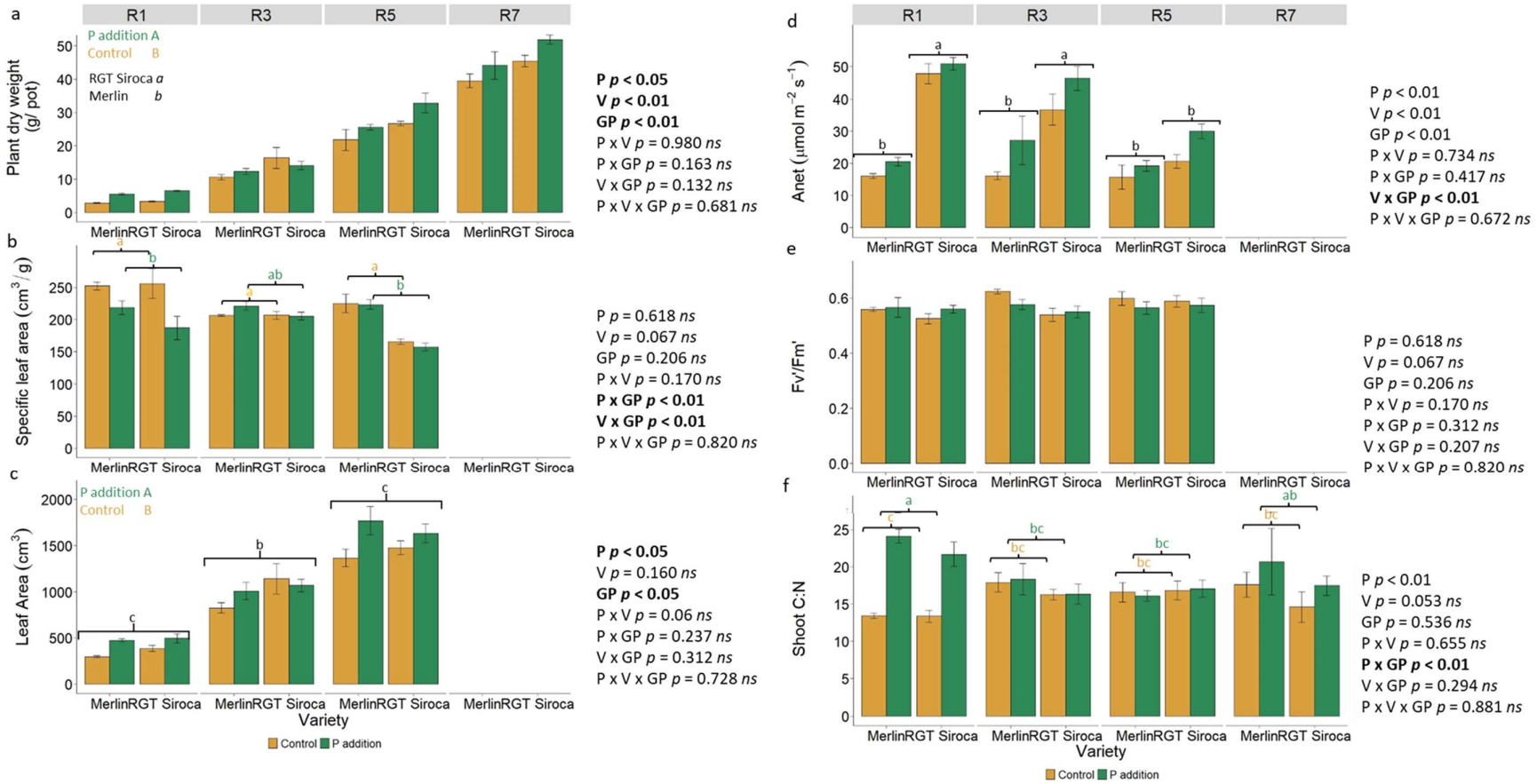


Figure 4.3. Plant morphological traits and photosynthetic performance of two varieties of soybean (Merlin and RGT Siroca) grown under two phosphorus (P) treatments (control and P fertiliser addition). Individual plants in pots were harvested at four distinct phases of phenological developments (R1, beginning of flowering; R3, beginning of pod formation; R5, beginning of pod filling; and R7, beginning of full maturity). Data are means ± SE of 6 replicates. Three-way ANOVA *p* values are displayed on the right of the figure, with letters of significance indicating results of post-hoc tests from ANOVA results shown in bold. Colours represent P treatment**. Figure 4.3.a.** plant dry weight (g) **Figure 4.3.b.** specific leaf area ($cm^3\ g^{-1}$) **Figure 4.3.c.** leaf area (cm^3) **Figure 4.3.d.** Net photosynthesis (µmol CO_2 m^{-2} s^{-1}) **Figure 4.3.e.** Light-adapted Photosystem II maximum efficiency **Figure 4.3.f.** shoot carbon (C): nitrogen (N) ratio.

4.3.4. Nodule trait and nitrogen fixation relationships

Across all phases of phenological developments, varieties and P treatments, RAU was significantly ($p < 0.001$) correlated ($r = 0.644$) with leghaemoglobin concentration (Figure 4.4). Relative abundance of ureides was also significantly ($p < 0.05$) correlated with nodule number, dry weight, average nodule dry weight and specific nodule dry weight, although these were much weaker correlations (r = 0.152, 0.217, 0.183 and 0.330, respectively). Shoot N was significantly ($p < 0.001$) correlated with nodule dry weight and nodule number (r = 0.910 and 0.844, respectively). Average nodule dry weight and specific nodule dry weight were also significantly ($p < 0.001$) correlated with shoot N (r = 0.675 and 0.672, respectively). Leghaemoglobin was not significantly ($p = 0.854$) correlated with shoot N ($r = 0.199$).

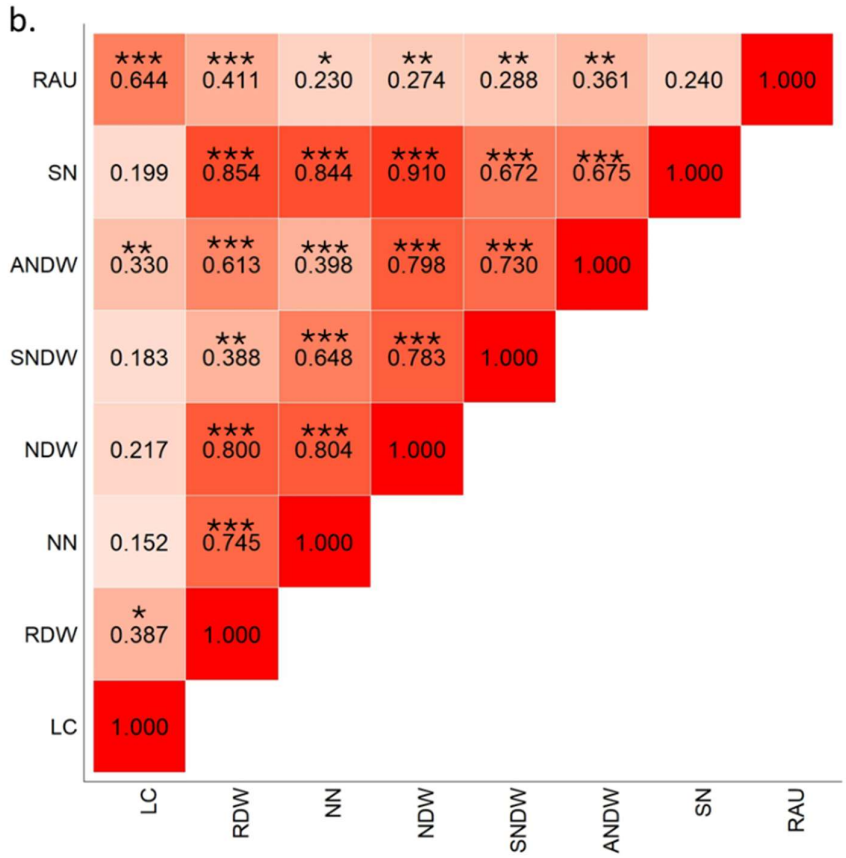


Figure 4.4. Spearman's rank correlation between measured nodule traits, root dry weight and nitrogen (N_2) fixation parameters across both varieties at all measured phases of phenological development and both phosphorus (P) fertiliser treatments. Pearson's correlation coefficient (r) is displayed within the squares with darker red indicating a stronger correlation, stars above represent significance (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$). Abbreviations are as follows: LC, leghaemoglobin concentration; RDW, root dry weight; NN, nodule number; NDW, nodule dry weight; SNDW, specific nodule dry weight; ANDW, average nodule dry weight; SN, shoot nitrogen content; RAU, relative abundance of ureides.

Analysis of covariance results found a significant interaction ($p < 0.001$) of leghaemoglobin and phenological development on RAU (Table 4.2). The relationship between leghaemoglobin and RAU is highly dependent on phase of phenological development, with RAU showing a visible increase following an increase in leghaemoglobin concentration at the R1 and R3 phase of phenological development, maximum RAU at all leghaemoglobin concentrations occurring at R5, and decreasing RAU with increasing leghaemoglobin concentration at the R7 phase of phenological development (Figure 4.5.a). When looking at linear regression across the different phase of phenological developments R^2 values were relatively low ($R^2 < 0.220$) and non-significant ($p > 0.05$), indicating that whilst there is a significant interaction in the ANCOVA, leghaemoglobin concentration alone is not a strong predictor of RAU when accounting for differences in phase of phenological development.

Analysis of covariance results found a significant interaction ($p < 0.05$) of nodule dry weight, variety and phase of phenological development on shoot N content (Table 4.3). Consequently, linear regression was performed for both varieties at different phase of phenological developments (Figure 4.5.b). When comparing the linear regression across the two varieties at different growth stages, the R1 stage of both varieties showed high and significant R^2 values ($R^2 = 0.759$ and 0.850, $p < 0.01$, for Merlin and RGT Siroca, respectively). Similar slopes were evident for the shoot N versus nodule dry weight regression lines, indicating nodule dry weight increasing shoot N proportionally for both varieties. At the R3 phase of phenological development a similar response is evident, with slightly lower R^2 values ($R^2 = 0.585$ and 0.521 for Merlin and RGT Siroca, respectively). When comparing the slopes of the shoot N versus nodule dry weight regression lines, the R1 phase of phenological development increased shoot N 1.5-fold compared to the R3 phase of phenological development. At the R5 phase of phenological development, only Merlin had a significant R^2 value ($R^2 = 0.713$, $p < 0.01$), with RGT Siroca not showing a significant linear regression. At the R7 phase of phenological development, neither variety displayed significant regression, indicating that there is no longer a significant relationship between nodule dry weight and shoot N. An ANCOVA showed no significant interactions with nodule number (Table 4.3), and nodule number significantly increased shoot N, regardless of variety, P treatment or phase of phenological development (Figure 4.5.c, $R^2 = 0.617$, $p < 0.001$). For leghaemoglobin, nodule dry weight and nodule number there was no significant ANCOVA interaction between nodule trait and phosphorus treatment ($p = 0.748$, 0.263 and 0.498, respectively).

Table 4.2. Summary of *p* values from analysis of covariance (ANCOVA) assessing the effects of nodule leghaemoglobin concentration, phosphorus treatment, variety and phase of phenological development on relative abundance of ureides.

Source of Variation	ANCOVA *p* value
Leghaemoglobin (L)	< 0.001
Phosphorus (P)	0.158
Variety (V)	0.945
Phase of phenological development (GP)	< 0.001
L x P	0.748
L x V	0.152
L x GP	< 0.001
P x V	0.083
P x GP	< 0.001
V x GP	< 0.001
L x P x V	0.338
L x P x GP	0.782
L x V x GP	0.436
P x V x GP	< 0.001
L x P x V x GP	0.934

Table 4.3. Summary of *p* values from ANCOVA analysis assessing the effects of nodule dry weight or nodule number, phosphorus treatment, variety and phase of phenological development on shoot nitrogen content.

Source of Variation	ANCOVA *p* value	Source of Variation	ANCOVA *p* value
Nodule dry weight (NDW)	< 0.001	Nodule number (NN)	< 0.001
Phosphorus (P)	< 0.05	Phosphorus (P)	< 0.001
Variety (V)	< 0.05	Variety (V)	< 0.01
Phase of phenological development (GP)	< 0.001	Phase of phenological development (GP)	< 0.001
NDW x P	0.263	NN x P	0.498
NDW x V	0.053	NN x V	0.413
NDW x GP	0.882	NN x GP	0.135
P x V	0.866	P x V	0.412
P x GP	< 0.01	P x GP	0.208
V x GP	< 0.01	V x GP	0.783
NDW x P x V	0.608	NN x P x V	0.722
NDW x P x GP	0.388	NN x P x GP	0.664
NDW x V x GP	< 0.05	NN x V x GP	0.286
P x V x GP	0.450	P x V x GP	< 0.01

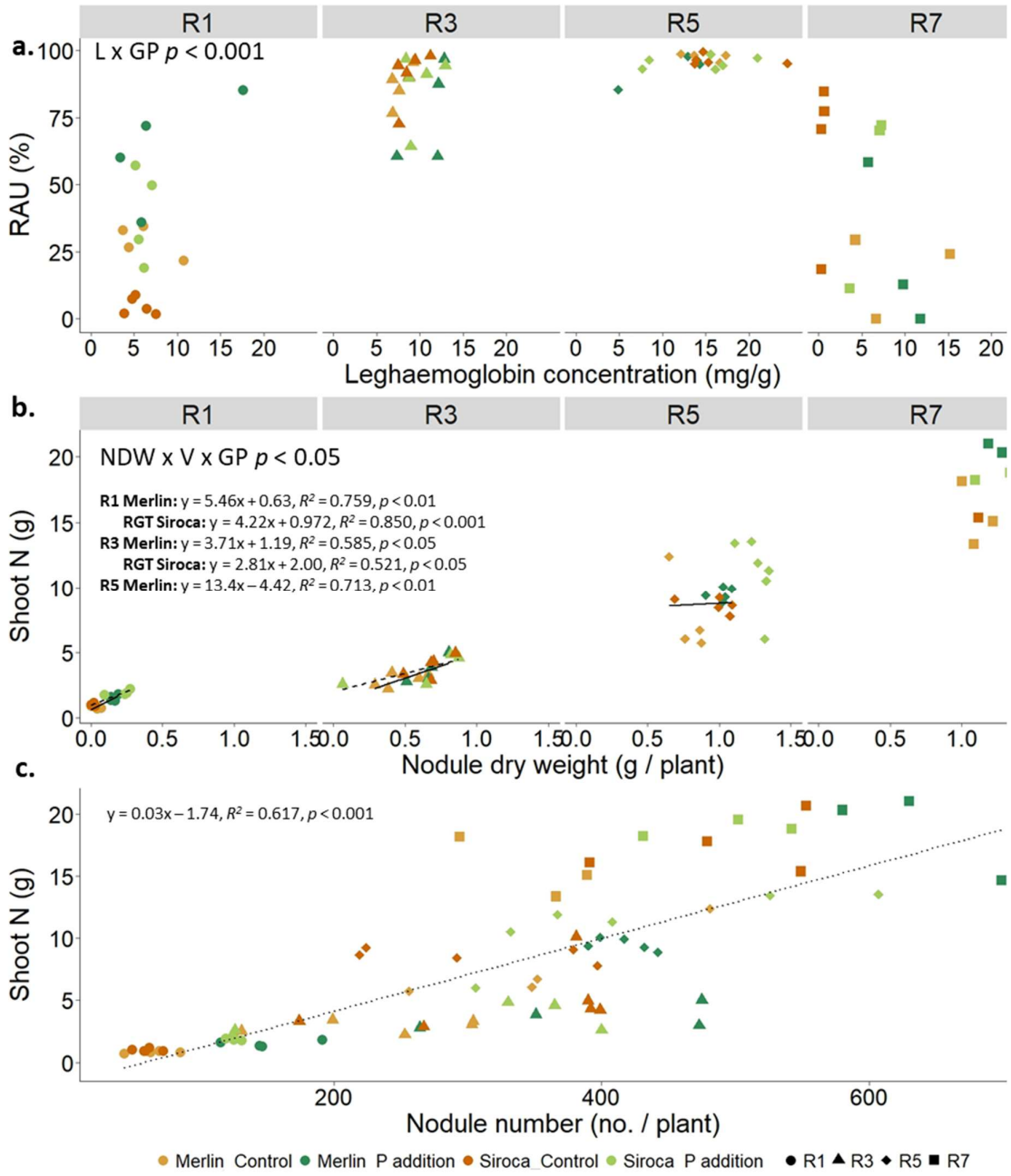


Figure 4.5. Measures of nitrogen fixation plotted against key nodule traits. Colours represent phosphorus (P) treatment and variety, and shape represents phenological development. **Figure 4.54.a.** Relative abundance of ureides (RAU) plotted against nodule leghaemoglobin concentration at individual phases of phenological development **Figure 4.5.b.** Shoot nitrogen (N) plotted against nodule dry weight at individual phases of phenological development **Figure 4.5.c.** Shoot N plotted against nodule number across the full growth cycle. Linear models were fitted based on significant interactions with respective nodule trait, with significant trend lines displayed on the graphs and equations, R^2 and p values listed to the left. The displayed p values are the significant interaction terms from ANCOVA analysis (Table 4.2 and 4.3).

4.3.5. Yield response to phosphorus addition

A significant ($p < 0.01$) 25% increase in seed yield following P fertiliser addition compared to the control treatment was observed, with no significant varietal response or P x variety interaction (Figure 4.6.a). A similar response was observed when looking at HI, with both varieties showing a significant ($p < 0.05$) 10% increase following P fertiliser addition (Figure 4.6.b). Pod number per plant increased significantly ($p < 0.01$) following P fertiliser addition (Table 4.4). Thousand seed weight showed a 7% trend increase following P fertiliser addition, but this was not significant ($p = 0.058$). No significant ($p = 0.214$) difference was observed following P fertiliser addition in the number of seeds per pod.

Both pod number per plant and seed number per plant at the R7 phase of phenological development increased significantly ($p < 0.01$) following P fertiliser addition (Table 4.4). Thousand seed weight showed a 7% trend increase following P fertiliser addition, but this was not significant ($p = 0.058$). No significant ($p = 0.214$) difference was observed in the number of seeds per pod at the R7 phase of phenological development. Merlin had a significantly ($p < 0.01$) greater HI compared to RGT Siroca, but no significant ($p = 0.358$) P x variety interaction was observed (Figure 4.5.b). Whilst RGT Siroca had a significantly ($p < 0.001$) greater thousand seed weight, by 20%, than Merlin, Merlin had a significantly ($p < 0.001$) greater seed number per plant and seed number per pod, with seed number increasing by 23 and 19% respectively (Table 4.4).

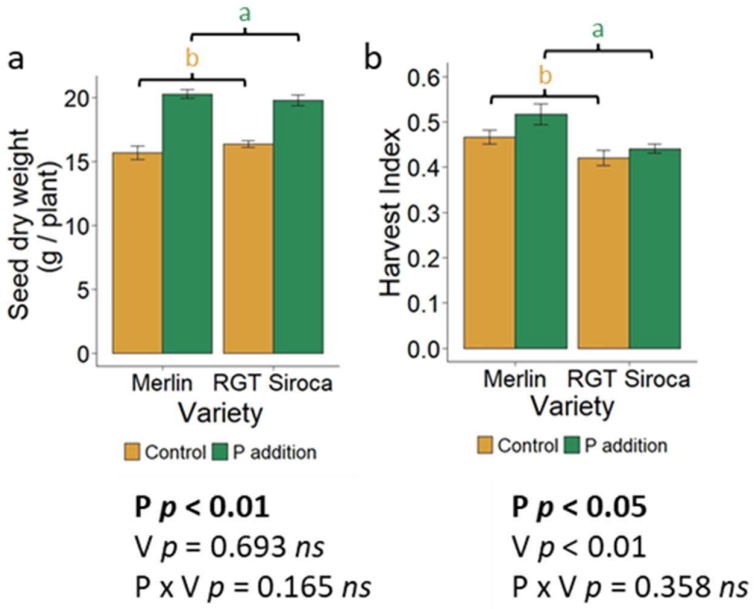


Figure 4.6. Yield response of two varieties of soybean (Merlin and RGT Siroca) grown under two phosphorus treatments (control and phosphorus fertiliser addition). Data are means ± SE of 6 replicates. Two-way ANOVA p values are displayed on the bottom of the figure, with letters of significance indicating results of post-hoc tests from ANOVA results shown in bold. Colours represent phosphorus treatment. **Figure 4.6.a.** seed yield at the beginning of maturity **Figure 4.6.b.** harvest index at the beginning of maturity.

Table 4.4. Yield traits of two varieties (Merlin and RGT Siroca) of soybean grown under two phosphorus (P) treatments (control and P fertiliser addition). Individual plants in pots were harvested at the R7 phase of phenological development). Data are means ± SE of 6 replicates. Two-way ANOVA p values are displayed.

Source of Variation		Pod number (no. plant $^{-1}$)	1000 seed weight (g)	Seed number (no. pod^{-1})	Pod HI
Variety	**Phosphorus**				
Merlin	Control	43.5 ± 2.6	167 ± 8	2.2 ± 0.1	0.66 ± 0.01
Merlin	P fertiliser addition	49.6 ± 1.9	178 ± 8	2.3 ± 0.1	0.68 ± 0.01
RGT Siroca	Control	42.5 ± 0.9	200 ± 4	1.9 ± 0.1	0.67 ± 0.01
RGT Siroca	P fertiliser addition	48.6 ± 1.5	216 ± 5	1.9 ± 0.1	0.68 ± 0.01
	Phosphorus (P)	< 0.01	0.058	0.214	0.066
	Variety (V)	0.603	< 0.001	< 0.001	0.436
	P x V	1.00	0.716	0.178	0.348

4.4. Discussion

4.4.1. Influence of phosphorus fertiliser on nitrogen fixation

The significantly higher RAU in the R1 phase of phenological development under P fertiliser addition, and the increase in shoot N content across multiple phases of phenological developments, provide further evidence that P fertiliser addition increases N_2 fixation (Figure 4.1). The 21-step mechanism involved in the fixation process of atmospheric N to NH_3 is highly energy intensive, requiring large amounts of phosphorus containing ATP (Baral et al., 2016). Alongside the direct energy demands for the process of N_2 fixation, P fertiliser addition indirectly drives N_2 fixation through the allocation of resources and photosynthate below-ground to drive nodule tissue formation and maintenance, allowing for increased sites for N_2 fixation to occur.

Phosphorus addition increased nodule number and dry weight (Figure 4.2.a and Figure 4.2.b), particularly at the R1 phase of phenological development. At the R1 phase of phenological development, an increase in nodule number was observed, likely due to increased nodule formation in earlier phase of phenological developments following P fertiliser addition. Nodule formation is driven by two primary processes, one leading to the formation of infection sites, and the second leading to nodule morphogenesis (Downie, 2014). These programmes are driven by several regulatory mechanisms, including the interconnected effects of plant synthesised flavonoids coupled with the perception of rhizobia produced nodulation factors coordinating the attachment of free-living rhizobia to root hairs (Baral et al., 2016; Liu and Murray, 2016). Phosphorus deficiency has been demonstrated to negatively affect these early symbiotic events in common bean (Isidra-Arellano et al., 2018). Evidence suggests that the downregulation of nodule formation under low P supply is related to a feedback mechanism involving asparagine accumulation in nodules (Cabeza et al., 2024). This asparagine can be translocated to the shoots, leading to decreased C:N ratio (observed in the significantly lower shoot C:N ratio under control conditions at the R1 phase of phenological development, Figure 4.3.f.), repressing nodule activity or the formation of new nodules (Voisin et al., 2003).

Nodule number continues to increase under P fertiliser addition at later growth stages, as plant growth continues (Figure 4.2.a.). The continued regulation of nodule number following original infection is regulated by hormone signalling, the autoregulation of nodulation (AON), currently understood as a systematic long-range root and shoot signalling process (Nishida and Suzaki, 2018). Evidence in common bean indicates P deficiency inhibits additional nodule formation at later stages in the growth cycle through the activation of critical components of the AON pathway (Isidra-Arellano et al., 2018). The regulation of nodule number at later phase of phenological developments is also indirectly influenced by environmental factors controlling plant growth through the availability of assimilates for allocation to rhizobial symbiosis (Voisin et al., 2013).

At the R1 phase of phenological development there is a trend (though not statistically significant) in increased leghaemoglobin concentration within the nodules following P

fertiliser addition (Figure 4.2.e.). Sufficient P rises the ATP:ADP ratio within the nodules, indicating increased energy availability and increased nodule activity through improved functioning of nitrogenase enzymes, suggesting increasing leghaemoglobin concentration to buffer the free O_2 concentration to maintain increased activity (Cabeza et al., 2024). However, by quantifying leghaemoglobin concentration throughout the growth cycle following P fertiliser addition, differences in nodule activity were not significant (Figure 4.2.e.). The random sub-sampling for leghaemoglobin concentration may not have effectively encapsulated real-time nodule activity, as throughout the growth period the activity of individual nodules fluctuates depending on age and location on the root, resulting in varying levels of leghaemoglobin concentration. Active nodules contain four major and minor leghaemoglobins that can be observed in the internal red colour; prior to becoming active, nodules have an internal white colour, and during nodule senescence a green colour is observed due to the reaction of leghaemoglobin with nitrite in the mild acid pH of senescent nodules (Larrainzar et al., 2020). Whilst not quantified throughout this experiment, variations in nodule colour and hence nodule activity varied depending on location on the root and an increase in green nodules due to nodule senescence observed at later phase of phenological developments.

Whilst P fertiliser addition increased several nodule traits associated with improved N_2 fixation, P fertiliser addition did not influence the relationship between nodule traits and N_2 fixation (Table 4.3). Nodule dry weight was positively correlated with shoot N mass at the R1, R3 and R5 phase of phenological development (Figure 4.4.b) and nodule number was positively correlated with shoot N mass (Figure 4.4.c), regardless of P treatment or variety. Whilst P fertiliser addition stimulates increased nodulation, the relationship between nodulation and N_2 fixation remaining stable suggests P fertiliser addition does not improve the efficiency of N_2 fixation per nodule. This response has been seen in other studies, where P fertiliser addition increased nodulation rather than N_2 fixation efficiency(Li et al., 2022a; Li et al., 2021a).

The non-significant response of leghaemoglobin concentration to P fertiliser addition (Figure 4.2.e), coupled with the lack of significance in RAU between P treatments at the R3, R5 and R7 phase of phenological development (Figure 4.1.a) may be a potential adaption to P deficiency as growth continues. Nodulated plants have evolved a number of adaptive strategies to combat P deficiency, including maintenance of P concentration in nodules; increasing P acquisition; and increasing N_2 fixation per unit of nodule mass and higher nodule permeability (Sulieman and Tran, 2015). Alternative mechanisms to conserve P have been suggested, such as the increase of phosphohydrolase enzyme activity, recycling of membrane phospholipids and the use of alternative biochemical pathways (Vardien et al., 2016). The P concentration in nodules can also be upregulated by transporter genes implicated in direct P uptake (Qin et al., 2012). A potential mechanism to be explored in future research is the maintenance of optimal nodule P status through differentiated P partitioning and metabolism within plants across the growth period. Better mechanistic understanding of the response and adaption of soybean-rhizobia symbiosis to P deficiency, through the study of metabolic pathways or

plant hormone signalling, will aid our ability to mitigate P deficiency and potentially reduce reliance on P fertiliser use.

The non-significant response in RAU between P treatments at the R3, R5 and R7 phase of phenological development (Figure 4.1.a.) may also be due to the low available N content of the sandy growth substrate (Table 4.1.) necessitating demand for large proportion of plant N to come from fixation processes as plant growth continues and soil available N stocks are used. To achieve these N_2 fixation requirements under low N and P conditions, the recovery in RAU may be resultant from a trade-off for other growth parameters. For example, carbohydrates derived from photosynthesis are largely invested in below ground plant parts, often inhibiting whole plant growth (Zhu et al., 2023). This can be observed in the proportionally lower rates of photosynthesis and above-ground plant growth at these later phases of phenological development (Figure 4.3). The decline in RAU at the R7 phase of phenological development corresponds with the widely recognised decline of active fixation as leaves begin to senesce and discontinue photosynthesis (Hanway and Weber, 1971; Mastrodomenico and Purcell, 2012). Despite a significant decline in RAU, Merlin continues to increase nodule number under P fertiliser addition at the R7 phase of phenological development. This continued allocation to nodule formation may be a compensatory mechanism by the plant, attributed to the role of P in supporting residual N_2 fixation as nodule activity declines to try and optimise nutrient acquisition in preparation for seed filling (Yao et al., 2022).

4.4.2. Influence of phosphorus fertiliser on production

Phosphorus fertiliser addition significantly increased both seed yield and HI, by 25% and 10% respectively (Figure 4.6). These results confirm the importance of sufficient P fertiliser use to optimise soybean production and aid in the closure of yield gaps to meet growing demand. A conceptual diagram visualising the response of key parameters examined in this study to P fertiliser addition can be seen in Figure 4.6.

Both overall seed number and pod number per plant significantly ($p < 0.001$) increased following P fertiliser addition. Previous research suggests seed number is highly correlated with seed yield in the majority of crops (including soybean), with the R3 – R6 phase of phenological development suggested as the most critical for seed number determination (Monzon et al., 2021). A 14% significant ($p < 0.01$) increase in pod number was observed following P fertiliser addition. Pod number is determined by node number (influenced by genotype, branching, plant height and internode length), and canopy photosynthesis and crop growth during flowering and pod set (Egli, 2013). Phosphorus fertiliser addition can mitigate stress, prolong vegetative growth, accelerate shoot growth and stimulate lateral branching to increase node number. The increase in node number coupled with significant increases in photosynthesis and crop growth following P fertiliser (observed in this study) during the R1 and R3 phase of phenological developments contribute to the increased pod number observed.

The 10% increase in HI following P fertiliser addition, suggests not only does P increase seed yield by increasing the resources available to be partitioned to the seed, but there

is also an increase in proportion of resources partitioned to the seed. This significant HI increase is thought to be because of the trend in increase in pod HI and thousand seed weight (3% increase, p = 0.066 and 7%, p = 0.058 respectively). The seed number per pod was not significantly increased by P fertiliser addition, so it appears under P fertiliser addition there is an increase in resources partitioned to the seed at grain filling. This may include more efficient remobilisation of photosynthate or nutrients from other plant parts, further research should be undertaken to determine how nutrients and photosynthate are mobilised within the plant at multiple phases of phenological development.

4.5. Conclusion

This chapter provides evidence that P fertiliser addition drives an increase in nodulation rather than increasing nodule function. Results showed that increased nodulation following P fertiliser addition, through increased nodule formation and nodule dry weight, drives the increase in N_2 fixation observed rather than improving nodule function. This is coupled with increased demand for N, following increased and maintained growth across the growth cycle following P fertiliser addition, rather than P influencing the relationship between nodulation and N_2 fixation. Phosphorus addition was found to have a lesser effect on nodule function, thought to be because of the combined effect of regulatory mechanisms to maintain nodule function and trade-off with above-ground plant growth to achieve sufficient N_2 fixation under low P soils. As such the first hypothesised pathway in which optimal P supplies will enhance N_2 fixation (P fertiliser addition will drive an increase in nodulation, through increasing photosynthetic capacity and resource accumulation and allocation) has been accepted. Collectively, the results highlight the importance of early season P nutrition on establishing improved mechanisms for N_2 fixation throughout the growth cycle, leading to an increase of 15% shoot N mass and 25% seed yield across both varieties. Phosphorus fertiliser addition improves biomass accumulation though improvements to photosynthesis and N_2 fixation, resulting in a significant increase in pod formation and trends of increased allocation to seed filling cumulating in a significant increase in seed yield.

5. Phosphorus partitioning and mobilisation within soybean under contrasting phosphorus fertiliser treatments

In the previous chapter the role of phosphorus (P) fertiliser addition on improving nodule formation and resultant nitrogen (N_2) fixation and production was examined. In this chapter, within plant P partitioning and mobilisation across the growth cycle is investigated.

5.1. Introduction

Soybean production is required to increase to meet global demand, with current yield gaps estimated to be approximately 30% (Di Mauro et al., 2018; Merlos et al., 2015; Sentelhas et al., 2015; Weiner, 2017). Whilst P fertiliser addition has been estimated to increase soybean yield by approximately 20% (Chapter 2 and 4, (Ran et al., 2024; Xu et al., 2024)), the non-renewable nature of the resource means P supply to agriculture should be used sustainably, with efforts required to reduce the use of P fertiliser. Improving crop P efficiency can be achieved through improving P acquisition efficiency (PAE, the ability of the crop to uptake P from soils) and/or P utilisation efficiency (PUE, the ability to produced yield using acquired P) (Wang et al., 2010). The plasticity of soybean root system means improving soybean uptake through modification in plant root morphology is one potential to improve PAE (Bello, 2021; Falk et al., 2020; Vance et al., 2003; Wang et al., 2021). Following P uptake, the partitioning and remobilisation of internal P to improve PUE acts as an understudied yet essential area where soybean P efficiency can be improved.

The evolution of management practices and cultivar breeding to optimise higher yielding soybean production has potential to alter patterns of nutrient uptake, partitioning and remobilisation studied during the 20th century. For example, at full maturity, R8, (Bender et al., 2015) reported a total P uptake of 21 kg P ha^{-1} for a 3480 kg ha^{-1} seed yield, and 81% of total P uptake partitioned to the seed, whereas (Hanway and Weber, 1971) reported an increased total P uptake of 22.6 kg P ha^{-1}, and a lower 72% of total P uptake partitioned to the seed, for a lower 3168 kg ha^{-1} seed yield. As a result, to ensure accurate P fertiliser guidelines and recommendations, the study of P dynamics in modern day soybean under current environment and management practices is required.

Early season P supply has been identified as critical for later growth and development, with supply during early development being found to have a dominating effect on crop potential through the promotion of P uptake and storage for reallocation at later stages (Grant et al., 2001; Lambers, 2022; López-Arredondo et al., 2014). The transition from root uptake driven nutrient supply to remobilisation from senescing tissues is vital for the fitness of a plant, particularly under stress conditions (Stigter et al., 2015). The transfer of nutrients from vegetative organs to developing seeds during senescence has been conserved in most major crop species through domestication and subsequent breeding programmes (Julia et al., 2016; Rose et al., 2012). Phosphorus is stored in large amounts in the soybean seed to improve seed vigour with seed quality has been shown to be positively correlated with P translocation efficiency (Zhao et al., 2023).

This chapter aims to investigate within plant P partitioning and mobilisation of two varieties of soybean across the growth period following contrasting early season P fertiliser treatments. The two varieties used, Merlin and RGT Siroca are two early maturity soybean varieties registered in Europe (000 and 00 maturity groups, respectively), with Merlin being indeterminate growth type registered in 1997 and RGT Siroca being semi-determinate registered in 2017 Austrian Federal Office for food Safety (2025). Whilst results of the previous chapter did not find significant ($p = 0.693$) differences in seed yield between the two varieties, Merlin had a significantly ($p < 0.001$) greater harvest index (HI) than RGT Siroca. Previous experimental work also found RGT Siroca to have significantly greater 1000 seed weight, seed yield and protein content but significantly lower oil content than Merlin (Omari et al., 2022). Merlin seed yield increased more following P fertiliser addition compared to RGT Siroca, 29.5 and 21% respectively (Figure 4.5). It is hypothesised that Merlin will also have more efficient remobilisation and partitioning of P during reproductive growth.

5.2. Methods

5.2.1. Experimental design and sampling

Two soybean genotypes (Merlin and RGT Siroca) were grown under controlled environment conditions in a randomised complete block design, with six replicates per treatment, under two P treatments (control and P fertiliser addition – 0 and 60 kg Pha^{-1}, respectively) with destructive harvesting at four distinct phase of phenological developments (R1 – beginning of flowering, R3 – beginning of pod formation, R5 – beginning of seed formation and R7 – beginning of full maturity). Four litre pots were filled with a 3:1 mixture of Leighton Buzzard lime free sand and sandy loam topsoil, to lower soil nutrient status. Characteristics of the sand: soil mix prior to fertiliser treatment are given in Table 4.1 and methods describing soil analysis completed are given in Section 9.2.6. When filling pots, granular magnesium and potassium fertilisers were applied to all pots at rates of 0.15 and 0.4 g pot^{-1}, respectively (18 and 86 kg ha^{-1} equivalent). Phosphorus fertiliser (single superphosphate, 18% P_2O_5) was applied at a rate of 0.75 g P pot^{-1} (equivalent to 60 kg P ha^{-1}) to the P addition treatments, with no P fertiliser being applied to the control treatment. Full details of experimental design and growth conditions can be seen in section 4.3.1.

5.2.2. Tissue sampling and analysis

At the four harvest stages, plants were partitioned to nodule, root, stem, leaf (including petioles), pod (R3 and R5 phase of phenological development including forming seeds, R7 excluding seeds) and seed (R7 phase of phenological development). At R7, fallen senesced leaves were excluding from leaf tissue analysis. Partitioned plant material was dried at 40°C for at least 72 h, weighed for dry weight then milled to <1 mm diameter using a ball mill. Approximately 0.25 g of tissue (or the minimum weight available) was digested in 5 mL nitric acid (HNO_3) using a CEM Mars 5 microwave with MARS Xpress vessels (CEM UK, United Kingdom). The samples were diluted to 20% HNO_3 for storage by adding 1 mL of digested sample to 4 mL of DI water. Samples were digested in twelve batches with standard reference material samples (Trace Elements in Spinach Leaves 1570a and Tomato Leaves 1573a,

National Institute of Standards and Technology, Maryland, USA) and digestion blanks (5 mL HNO_3) included in all batches to ensure for consistency between batches and methods and allow for limit of detection (LoD) and percentage recovery to be calculated.

All plant tissue digested sample was diluted further from 20% to 1% or 0.5% HNO_3 (determined based on expected tissue P content) and analysed for P concentration using the molybdenum blue colorimetry using a microplate reader (BMG Labtech, Ortenberg, Germany). Full methods for colorimetric determination of P concentration can be seen in Section 9.2.6.

Phosphorus stocks for each partitioned plant organ (mg plant^{-1}) were calculated by multiplying concentration (mg g^{-1}) by partitioned dry weight (g). Whole plant P stocks were calculated by adding partitioned P stocks together. Whole plant P concentration was calculated using weighted average mean concentration from partitioned P stocks.

Phosphorus harvest index (PHI) was calculated using the following equation:

$$PHI = \frac{Seed\ P\ (mg\ plant^{-1})}{Plant\ P\ (mg\ plant^{-1})}.$$

5.2.3. Macro- and Micro-nutrient analysis

The stored digested leaf tissue sample was diluted to 2% HNO_3 for analysis, by adding 1 ml of the 20% sample to 9 ml DI water and filtered through 0.45 µM syringe filter. A range of leaf tissue macro- (potassium (K), magnesium (Mg) and sulphur (S) and micro-nutrients (boron (B), calcium (Ca), copper (Cu), iron (Fe) and zinc (Zn)) were analysed using Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES; iCAP 6300, Thermo Scientific, Massachusetts, USA). The limit of detection (LoD, Table 5.1) was determined using the following equation:

$$LoD = Mean_{blank} + (3 \times SD_{blank})$$

Where $Mean_{blank}$ is the mean concentration of the 12 extraction blanks, and SD_{blank} is the standard deviation of the 12 extraction blanks. Manganese and molybdenum were analysed but have not been included within the analysis as the concentrations were below the calculated LoD. Mean % recovery across both standard reference material samples can be found in Table 5.2. Sample analysis was corrected, if required, using the % recovery and blank samples run.

Table 5.1. Limit of detection (LoD) of leaf tissue macro- and micro-nutrients analysed using Inductively Coupled Plasma - Optical Emission Spectrometry. Calculated from 12 extraction blanks.

	Macro-nutrients (ppm)		
	Magnesium	Potassium	Sulphur
LoD	0.009	0.015	0.187

	Micro-nutrients (ppb)						
	Boron	Calcium	Copper	Iron	Manganese	Molybdenum	Zinc
LoD	3.67	0.065	3.22	3.05	0.006	24.6	1.41

Table 5.2. Percentage recovery of leaf tissue macro- and micro-nutrients from two standard reference material samples (Trace Elements in Spinach Leaves 1570a and Tomato Leaves 1573a, National Institute of Standards and Technology, Maryland, USA) analysed using Inductively Coupled Plasma - Optical Emission Spectrometry. Calculated from 12 extracted standard materials.

	Boron	**Calcium**	**Copper**	**Iron**	**Potassium**	**Magnesium**	**Sulphur**	**Zinc**
Spinach leaves (1570a)	85.65 ± 2.78	87.08 ± 3.77	98.99 ± 6.98	-	81.37 ± 0.73	-	-	94.64 ± 4.48
Tomato leaves (1573a)	-	87.93 ± 0.96	-	83.06 ± 0.97	83.58 ± 1.50	-	-	109.66 ± 0.35

5.2.4. Experimental Design and Statistical analysis

Outliers were determined if greater than two standard deviations from the mean and removed from the dataset. Data were tested for normality using the Shapiro-Wilk test and histograms and Q-Q plots were visually assessed. Where data were not normally distributed, they were transformed using either logarithmic or square root functions to achieve normality. A three-way analysis of variance (ANOVA) assessed for statistical differences in plant response, with phase of phenological development, P treatment and genotype as factors. A two-way ANOVA assessed for statistical differences in plant response, with P treatment and genotype as factors. To assess pairwise differences following a significant three-way or two-way ANOVA interaction, Bonferroni post hoc tests were applied to control for Type 1 error across multiple comparisons. When only a significant main effect was detected without interactions, Tukey's HSD post hoc test was applied, employing a more powerful approach for multiple comparisons when interactions are not present.

5.3. Results

5.3.1. Plant phosphorus dynamics at maturity

Whole plant P stocks (mg P plant^{-1}) were significantly ($p < 0.001$) greater under P fertiliser addition compared to the control, with RGT Siroca having significantly ($p < 0.05$) greater whole plant P stocks than Merlin (Figure 5.1.a). Whilst no significant P x variety interaction was observed ($p = 0.265$), Merlin appeared to show a trend in increased response to P fertiliser addition with Merlin whole plant P stocks increasing by 93% under P fertiliser addition compared to the control, whereas RGT Siroca only increased by 34% following P fertiliser addition compared to the control. Whole plant P concentration across both varieties (mg P g^{-1}) was significantly ($p < 0.001$) greater following P fertiliser addition compared to the control (26% increase), with no significant varietal response ($p = 0.341$) observed (Figure 5.1.b). Seed P concentration (mg P g^{-1}) was significantly ($p < 0.001$) greater across both varieties following P fertiliser addition compared to the control, with no significant varietal response ($p = 0.216$) observed (Figure 5.1.c). Phosphorus harvest index did not show significant ($p = 0.292$), response to P fertiliser addition but Merlin showed a significantly greater ($p < 0.05$) PHI than RGT Siroca (Figure 5.1.d).

The two varieties had different biomass partitioning patterns at the R7 phase of phenological development (Figure 5.2.a). Merlin allocated significantly ($p < 0.01$) more biomass to the seed than RGT Siroca (41 and 36% respectively). Merlin also allocated significantly ($p < 0.01$) more biomass to the pod than RGT Siroca (21 and 17% respectively). The opposite pattern was observed for the leaf, with Merlin allocating significantly ($p < 0.01$) less biomass than RGT Siroca (9 and 17% respectively). The two varieties also had different P partitioning patterns at the R7 phase of phenological development (Figure 5.2.b). Merlin allocated significantly ($p < 0.001$) more P to the seed than RGT Siroca (81 and 72% respectively). Whilst not significant, there was a trend ($p = 0.08$) in increased P allocated to the pod in Merlin where it accounts for 5% compared to RGT Siroca (3.8%). The opposite pattern was observed for P partitioning

to the leaf with RGT Siroca allocating significantly (p <0.01) more P than Merlin (16 and 6% respectively).

At the R7 phase of phenological development, P partitioning patterns differed under different P treatments (Figure 5.2.b). Significantly (p < 0.05) more P was allocated to the seed following P addition (79%) compared with control (75%) treatments. The opposite pattern was observed for pod, whilst not significant there was a trend (p = 0.071) in increased P allocated to the pod under control P treatments where it accounts for 5.0% compared to P addition (3.5%). Phosphorus fertiliser treatment did not significantly affect biomass partitioning patterns (Figure 5.2.a).

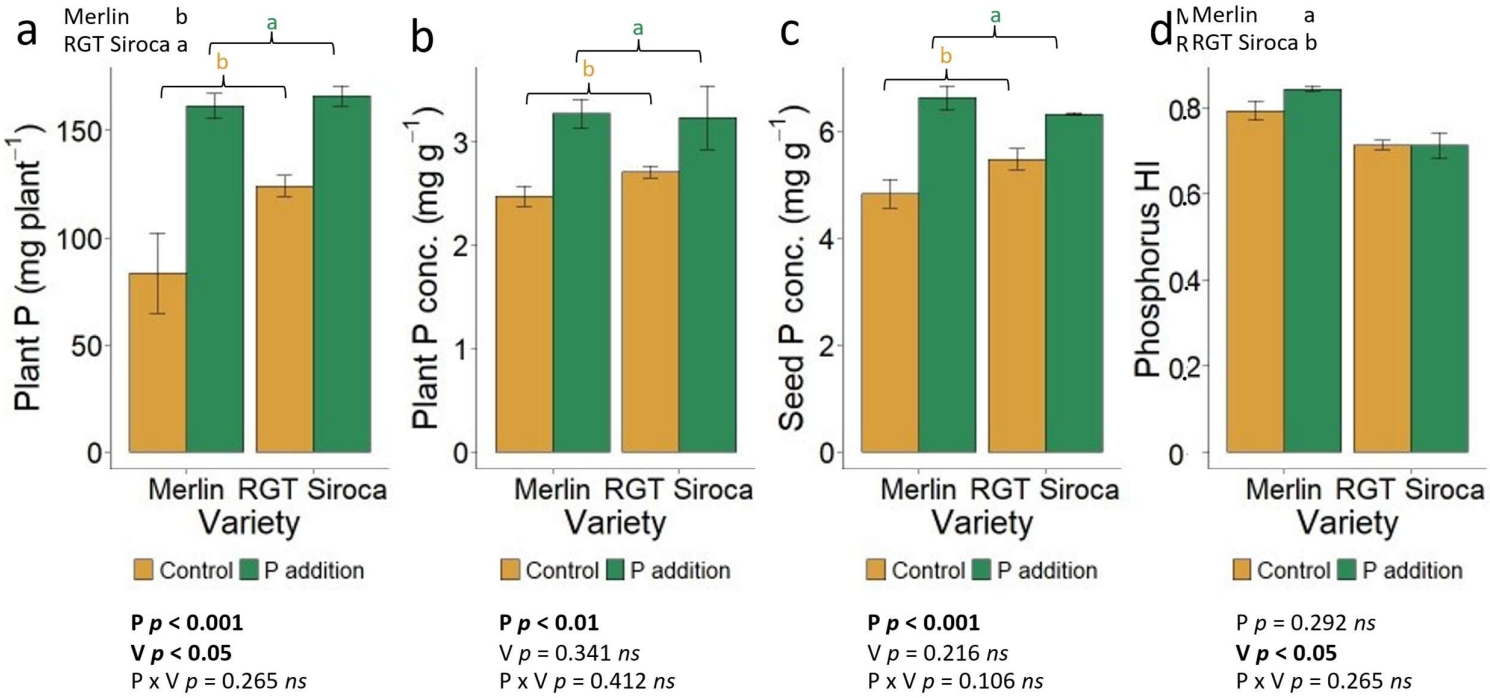
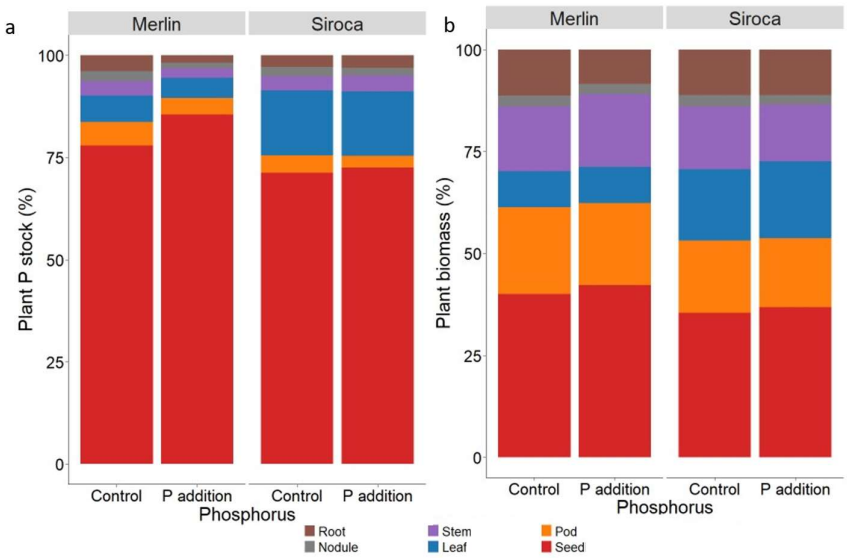


Figure 5.1. Phosphorus (P) behaviour at beginning of maturity (R7) of two varieties of soybean (Merlin and RGT Siroca) grown under two P treatments (control and P fertiliser addition). Data are means ± SE of 6 replicates. Two-way ANOVA *p* values are displayed at the bottom of the figure, with letters of significance indicating results of post-hoc tests from ANOVA results shown in bold. Colours represent phosphorus treatment. **Figure 5.1.a** Whole plant P stock (mg plant^{-1}), **Figure 5.1.b** whole plant P concentration (mg g^{-1}) **Figure 5.1.c** seed P concentration (mg g^{-1}), and **Figure 5.1.d** P harvest index (HI).



Source of variation	Nodule	Root	Stem	Leaf	Pod	Seed
Biomass partitioning						
P	0.75	0.381	0.852	0.731	0.256	0.350
V	0.867	0.579	0.234	< 0.01	< 0.01	< 0.01
P x V	0.663	0.401	0.222	0.711	0.918	0.845
Phosphorus partitioning						
P	0.242	0.247	0.495	0.757	0.071	< 0.05
V	0.995	0.787	0.510	< 0.01	0.084	< 0.001
P x V	0.553	0.166	0.222	0.803	0.802	0.119

Figure 5.2. Partitioning to organs of two varieties of soybean (Merlin and RGT Siroca) at beginning of maturity (R7) under contrasting phosphorus (P) treatments (control and P fertiliser addition). Data are means of 6 replicates. Colour represents plant organ. **Figure 5.2.a** plant P stock partitioning and **Figure 5.2.b** plant biomass partitioning. Two-way ANOVA *p* values are displayed at the bottom of the figure.

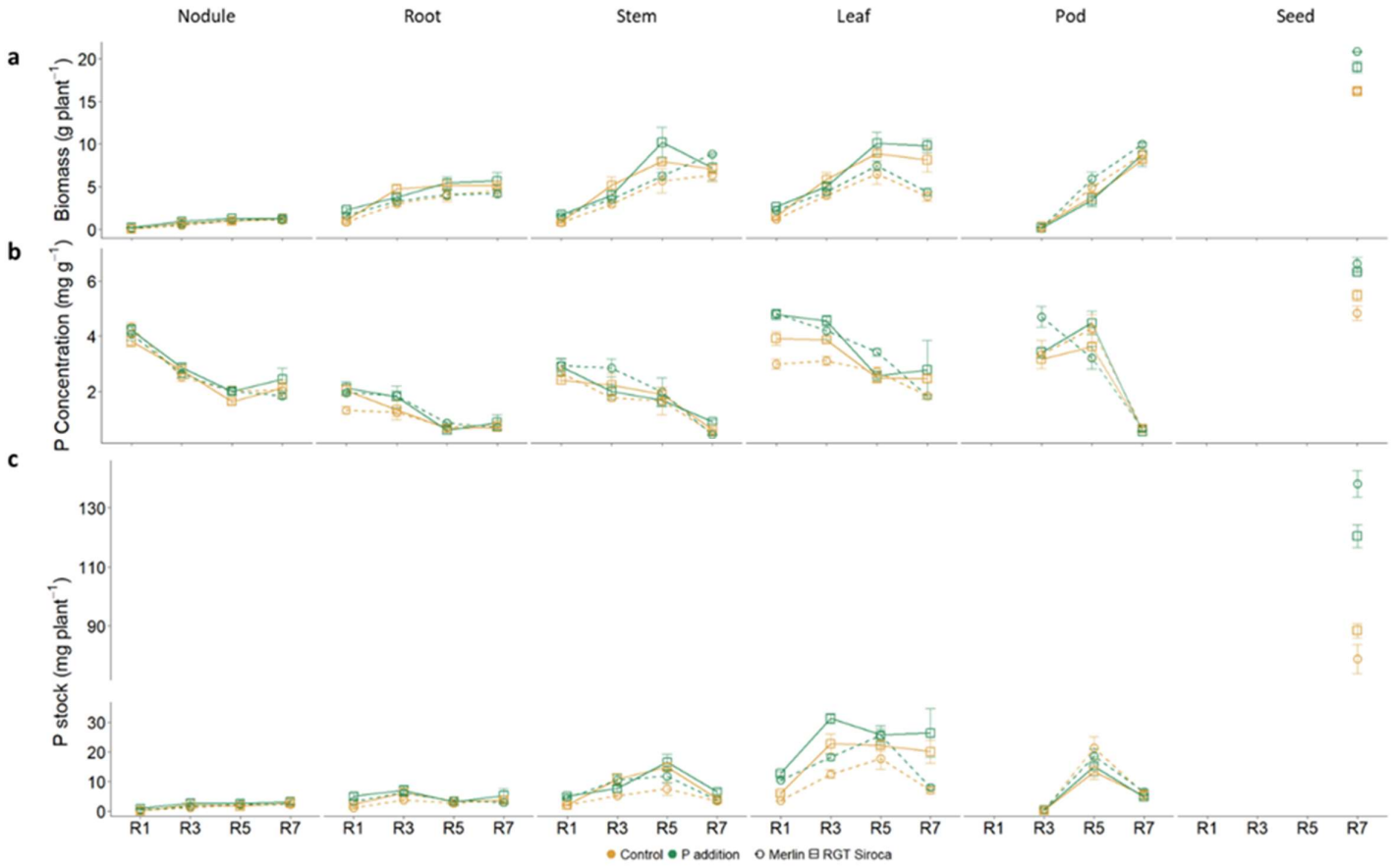


Figure 5.3. Partitioning amongst organs of soybean varieties (Merlin and RGT Siroca) at four different phases of phenological developments (R1, beginning of flowering; R3, beginning of pod formation; R5, beginning of pod filling; and R7, beginning of full maturity), grown under two phosphorus (P) treatments (control and P fertiliser addition). Individual plants in pots were harvested at four distinct phases of phenological development, data are means ± SE of 6 replicates. **Figure 5.3.a** biomass partitioning (g organ^{-1}), **Figure 5.3.b** P concentration (mg g^{-1}) and **Figure 5.3.c** P stock partitioning (g organ^{-1}). In Figure 5.3.c the y-axis has a discontinuity between 30 and 70 g, reflecting non-informative ranges to improve visualization. Colour represents P treatment, circles and dashed line represent Merlin and squares and continuous line represent RGT Siroca.

Table 5.3. Biomass (g plant^{-1}) of partitioned organs of two varieties of soybean (Merlin and RGT Siroca) grown under two P treatments (control and P fertiliser addition). Individual plants in pots were harvested at four distinct phases of phenological development (R1, beginning of flowering; R3, beginning of pod formation; R5, beginning of pod filling; and R7, beginning of full maturity). Data are means ± SE of 6 replicates. Three-way ANOVA *p* values are displayed.

Source of Variation									
Phase of phenological development (GP)	**Variety (V)**	**Phosphorus (P)**	**Leaf dry weight (g)**	**Stem dry weight (g)**	**Nodule dry weight (g)**	**Root dry weight (g)**	**Pod dry weight (g)**	**Seed dry weight (g)**	**Plant dry weight (g)**
R1	Merlin	Control	1.18 ± 0.07	0.76 ± 0.06	0.04 ± 0.01	0.83 ± 0.04	-	-	2.81 ± 0.07
R1	Merlin	P addition	2.20 ± 0.14	1.50 ± 0.08	0.16 ± 0.01	1.66 ± 0.23	-	-	5.59 ± 0.25
R1	Siroca	Control	1.52 ± 0.07	0.86 ± 0.06	0.02 ± 0.00	1.06 ± 0.17	-	-	3.36 ± 0.17
R1	Siroca	P addition	2.65 ± 0.06	1.70 ± 0.06	0.22 ± 0.03	2.29 ± 0.32	-	-	6.50 ± 0.19
R3	Merlin	Control	3.99 ± 0.29	2.98 ± 0.26	0.47 ± 0.07	2.98 ± 0.25	0.08 ± 0.03	-	10.60 ± 1.80
R3	Merlin	P addition	4.40 ± 0.31	3.55 ± 0.42	0.62 ± 0.05	3.29 ± 0.19	0.18 ± 0.10	-	12.30 ± 0.88
R3	Siroca	Control	5.85 ± 0.78	5.16 ± 1.00	0.75 ± 0.04	4.72 ± 0.56	0.37 ± 0.01	-	16.40 ± 3.14
R3	Siroca	P addition	5.02 ± 0.46	3.98 ± 0.59	0.95 ± 0.25	3.78 ± 0.26	0.17 ± 0.04	-	14.10 ± 1.31
R5	Merlin	Control	6.48 ± 1.21	5.68 ± 1.44	0.99 ± 0.25	3.92 ± 0.65	4.88 ± 0.50	-	21.80 ± 3.20
R5	Merlin	P addition	7.48 ± 0.52	6.28 ± 0.39	1.10 ± 0.05	4.08 ± 0.51	5.92 ± 0.77	-	25.60 ± 0.89
R5	Siroca	Control	8.89 ± 0.27	7.96 ± 0.27	0.97 ± 0.07	5.18 ± 0.18	3.74 ± 0.51	-	29.70 ± 0.65
R5	Siroca	P addition	10.09 ± 1.28	10.2 ± 1.79	1.27 ± 0.06	5.44 ± 0.70	3.41 ± 0.76	-	32.90 ± 3.00
R7	Merlin	Control	3.87 ± 0.58	6.30 ± 0.7	1.06 ± 0.04	4.63 ± 0.83	8.75 ± 0.71	16.32 ± 0.44	39.5 ± 2.15
R7	Merlin	P addition	4.39 ± 0.43	8.83 ± 0.14	1.23 ± 0.05	4.17 ± 0.29	9.97 ± 0.22	20.84 ± 0.04	44.2 ± 4.15
R7	Siroca	Control	8.14 ± 1.40	7.07 ± 0.53	1.28 ± 0.1	5.14 ± 0.23	8.16 ± 0.37	16.18 ± 0.34	45.40 ± 1.73
R7	Siroca	P addition	9.80 ± 0.78	7.25 ± 1.49	1.24 ± 0.15	5.68 ± 0.98	8.77 ± 1.34	19.03 ± 0.68	51.90 ± 1.37
		P	< 0.05	0.557	< 0.01	< 0.05	< 0.01	< 0.001	< 0.05
		V	< 0.001	< 0.001	0.658	< 0.001	< 0.05	<0.05	< 0.001
		GP	< 0.001	< 0.001	< 0.01	< 0.001	< 0.001	-	< 0.001
		P x V	0.332	0.443	0.595	0.914	0.291	0.165	0.980

P x GP	0.134	0.182	0.886	< 0.001	0.892	-	0.163
V x GP	< 0.001	<0.05	0.917	0.681	< 0.05	-	0.132
P x V x GP	0.740	0. 379	0.556	0.3587	0.856	-	0.681

Table 5.4. Phosphorus (P) concentration (mg g^{-1}) partitioned organs of two varieties of soybean (Merlin and RGT Siroca) grown under two P treatments (control and P fertiliser addition). Individual plants in pots were harvested at four distinct phases of phenological development (R1, beginning of flowering; R3, beginning of pod formation; R5, beginning of pod filling; and R7, beginning of full maturity). Data are means ± SE of 6 replicates. Three-way ANOVA *p* values are displayed.

Source of Variation									
Phase of phenological development (GP)	**Variety (V)**	**Phosphorus (P)**	**Leaf P (mg g^{-1})**	**Stem P (mg g^{-1})**	**Root P (mg g^{-1})**	**Nodule P (mg g^{-1})**	**Pod P (mg g^{-1})**	**Seed P (mg g^{-1})**	**Plant P (mg g^{-1})**
R1	Merlin	Control	2.99 ± 0.18	2.66 ± 0.25	1.32 ± 0.13	4.32 ± 0.30	-	-	2.42 ± 0.08
R1	Merlin	P addition	4.82 ± 0.16	2.93 ± 0.25	1.94 ± 0.08	4.07 ± 0.23	-	-	3.05 ± 0.18
R1	Siroca	Control	3.92 ± 0.25	2.4 ± 0.15	2.04 ± 0.19	3.81 ± 0.20	-	-	2.98 ± 0.04
R1	Siroca	P addition	4.78 ± 0.19	2.89 ± 0.29	2.12 ± 0.22	4.22 ± 0.19	-	-	3.44 ± 0.165
R3	Merlin	Control	3.1 ± 0.16	1.77 ± 0.12	1.24 ± 0.14	2.52 ± 0.14	3.33 ± 0.51	-	2.16 ± 0.06
R3	Merlin	P addition	4.19 ± 0.34	2.85 ± 0.32	1.84 ± 0.18	2.64 ± 0.15	4.69 ± 0.38	-	3.07 ± 0.08
R3	Siroca	Control	3.87 ± 0.1	2.23 ± 0.41	1.34 ± 0.36	2.78 ± 0.12	3.17 ± 0.32	-	2.59 ± 0.20
R3	Siroca	P addition	6.31 ± 0.34	2.00 ± 0.28	1.8 ± 0.39	2.86 ± 0.18	3.41 ± 0.19	-	2.94 ± 0.09
R5	Merlin	Control	2.72 ± 0.16	1.65 ± 0.5	0.7 ± 0.11	2.02 ± 0.17	4.27 ± 0.5	-	2.41 ± 0.13
R5	Merlin	P addition	3.42 ± 0.13	1.97 ± 0.53	0.85 ± 0.04	2.00 ± 0.12	3.22 ± 0.42	-	2.48 ± 0.09
R5	Siroca	Control	2.49 ± 0.18	1.88 ± 0.17	0.66 ± 0.12	1.93 ± 0.10	3.62 ± 0.47	-	2.07 ± 0.10
R5	Siroca	P addition	2.56 ± 0.07	1.68 ± 0.21	0.6 ± 0.13	2.00 ± 0.14	4.48 ± 0.42	-	2.08 ± 0.09
R7	Merlin	Control	1.83 ± 0.09	0.51 ± 0.08	0.81 ± 0.04	2.04 ± 0.27	0.67 ± 0.03	5.44 ± 0.77	2.47 ± 0.09
R7	Merlin	P addition	1.84 ± 0.03	0.42 ± 0.01	0.7 ± 0.09	1.83 ± 0.09	0.66 ± 0.1	6.12 ± 0.72	3.27 ± 0.14

R7	Siroca	Control	2.46 ± 0.12	0.62 ± 0.15	0.7 ± 0.03	2.12 ± 0.29	0.65 ± 0.14	5.47 ± 0.20	2.71 ± 0.06
R7	Siroca	P addition	2.78 ± 1.06	0.90 ± 0.10	0.87 ± 0.28	2.35 ± 0.38	0.55 ± 0.02	6.32 ± 0.02	3.23 ± 0.31
		P	< 0.001	< 0.001	< 0.001	0.125	< 0.05	< 0.05	< 0.001
		V	< 0.01	0.930	0.577	0.253	0.233	0.887	0.254
		GP	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	-	< 0.001
		P x V	<0.05	0.172	0.564	0.206	0.686	0.898	< 0.01
		P x GP	< 0.05	0.702	0.360	0.088	0.208	-	< 0.001
		V x GP	< 0.001	0.897	0.269	0.128	0.168	-	< 0.01
		P x V x GP	0.485	0. 202	0.514	0.678	0.075	-	0.366

Table 5.5. Phosphorus (P) stocks (mg plant^{-1}) partitioned organs of two varieties of soybean (Merlin and RGT Siroca) grown under two P treatments (control and P fertiliser addition). Individual plants in pots were harvested at four distinct phases of phenological development (R1, beginning of flowering; R3, beginning of pod formation; R5, beginning of pod filling; and R7, beginning of full maturity). Data are means ± SE of 6 replicates. Three-way ANOVA *p* values are displayed.

Source of Variation									
Phase of phenological development (GP)	**Variety (V)**	**Phosphorus (P)**	**Leaf P (g plant^{-1})**	**Stem P (g plant^{-1})**	**Root P (g plant^{-1})**	**Nodule P (g plant^{-1})**	**Pod P (g plant^{-1})**	**Seed P (g plant^{-1})**	**Plant P (g plant $^{-1}$)**
R1	Merlin	Control	3.49 ± 0.13	2.07 ± 0.33	1.10 ± 0.13	0.12 ± 0.04	-	-	6.43 ± 0.46
R1	Merlin	P addition	10.52 ± 0.43	4.37 ± 0.34	3.18 ± 0.36	0.52 ± 0.14	-	-	18.59 ± 0.54
R1	Siroca	Control	5.99 ± 0.55	2.07 ± 0.18	2.20 ± 0.47	0.06 ± 0.02	-	-	10.32 ± 0.63
R1	Siroca	P addition	12.69 ± 0.69	4.98 ± 0.64	5.02 ± 1.06	0.92 ± 0.12	-	-	23.61 ± 1.39
R3	Merlin	Control	12.5 ± 1.39	5.17 ± 0.30	3.66 ± 0.40	1.16 ± 0.15	0.32 ± 0.12	-	22.81 ± 1.48
R3	Merlin	P addition	18.11 ± 1.00	10.43 ± 1.97	6.12 ± 0.80	1.64 ± 0.22	0.64 ± 0.43	-	36.95 ± 2.93
R3	Siroca	Control	22.76 ± 3.21	10.83 ± 1.98	6.23 ± 1.56	2.09 ± 0.12	0.23 ± 0.23	-	42.14 ± 5.29
R3	Siroca	P addition	31.27 ± 1.71	7.60 ± 1.08	6.97 ± 1.75	2.72 ± 0.76	0.43 ± 0.17	-	49.00 ± 2.70
R5	Merlin	Control	17.76 ± 3.71	7.56 ± 2.22	2.58 ± 0.37	1.96 ± 0.49	21.37 ± 3.65	-	51.23 ± 4.52

R5	Merlin	P addition	25.61 ± 2.22	11.79 ± 2.25	3.42 ± 0.25	2.02 ± 0.19	18.45 ± 2.50	-	61.46 ± 1.29
R5	Siroca	Control	22.12 ± 1.81	14.85 ± 0.97	3.39 ± 0.60	1.59 ± 0.17	13.31 ± 2.46	-	55.26 ± 1.77
R5	Siroca	P addition	25.71 ± 3.15	16.46 ± 2.75	3.09 ± 0.69	2.54 ± 0.17	15.13 ± 3.26	-	62.96 ± 7.60
R7	Merlin	Control	7.01 ± 1.08	3.36 ± 0.72	3.83 ± 0.78	2.20 ± 0.37	5.91 ± 0.64	78.92 ± 4.95	83.51 ± 8.75
R7	Merlin	P addition	8.06 ± 0.66	3.72 ± 0.03	2.87 ± 0.15	2.26 ± 0.20	6.52 ± 0.85	138.06 ± 4.42	161.50 ± 5.92
R7	Siroca	Control	20.07 ± 3.9	4.17 ± 0.72	3.58 ± 0.29	2.74 ± 0.51	5.22 ± 1.06	88.44 ± 2.45	124.22 ± 4.93
R7	Siroca	P addition	26.37 ± 8.26	6.45 ± 0.82	4.21 ± 0.45	3.09 ± 0.86	4.77 ± 0.56	120.35 ± 3.83	166.24 ± 4.65
		P	< 0.01	< 0.001	< 0.01	< 0.001	0.374	< 0.05	< 0.001
		V	< 0.001	< 0.001	< 0.05	<0.01	0.495	0.120	< 0.001
		GP	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	-	< 0.001
		P x V	0.167	0.073	0.944	0.130	0.383	0.741	0.071
		P x GP	0.179	0.050	0.214	0.498	0.949	-	< 0.001
		V x GP	< 0.01	0.188	0.596	0.266	0.258	-	0.178
		P x V x GP	0.073	0.098	0.390	0.293	0.635	-	0.729

5.3.2. Remobilisation and partitioning across the growth cycle

Biomass

Leaf dry weight increased to the R5 phase of phenological development before decreasing at R7, with Merlin and RGT Siroca decreasing by 46 and 16% respectively (Figure 5.3.a). A significant variety x phenological development interaction ($p < 0.001$) was observed, with Bonferroni post hoc tests revealing RGT Siroca had significantly ($p < 0.001$) greater leaf dry weight than Merlin within the R5 and R7 phase of phenological development. Leaf dry weight was significantly ($p < 0.05$) greater following P fertiliser addition in both varieties at all phases of phenological developments. Stem dry weight increased to the R5 phase of phenological development in both varieties, before Merlin continuing to increase by 6% and RGT Siroca decreasing by 23%. A significant ($p < 0.05$) variety x phenological development interaction was observed, with RGT Siroca at the R5 phase of phenological development having a significantly ($p < 0.05$) greater stem biomass than Merlin.

Root dry weight increased from R1 to R3 then remained constant to the R7 phase of phenological development. A significant ($p < 0.01$) P x phenological development interaction was observed, with root dry weight being significantly ($p < 0.01$) lower in the control than P fertiliser addition at R1, control P being significantly lower than the P addition treatment at the R1 phase of phenological development, with all P treatments at the R3, R5 and R7 stages of phenological development being significantly greater than the R1 (with no significance between treatments).

For all other partitioned plant organs, no significant interactions were observed. Nodule dry weight was significantly ($p < 0.001$) greater with increasing phase of phenological development, and significantly ($p < 0.01$) greater following P fertiliser addition. Pod dry weight was significantly ($p < 0.01$) greater with increasing phase of phenological development, and significantly ($p < 0.01$) greater following P fertiliser addition.

Phosphorus concentration

Leaf P concentration remained constant from R1 to R3 then decreased from the R3 to R7 phase of phenological development (Figure 5.3.b). A significant ($p < 0.001$) variety x phenological development interaction was observed, with Merlin decreasing leaf P concentration between R5 and R7 by 40%, and RGT Siroca maintaining leaf P concentration (2% increase), hence RGT Siroca having a significantly ($p < 0.001$) greater leaf P concentration than Merlin at the R7 phase of phenological development (Table 5.4). A significant ($p < 0.05$) P x variety interaction was observed with Merlin under control P treatment (2.68 mg P g^{-1}) having significantly ($p < 0.05$) lower leaf P concentration than Merlin following P addition (3.92 mg P g^{-1}), and RGT Siroca under both P treatments (3.22 and 3.92 mg P g^{-1} respectively), and RGT Siroca under control P treatment having a significantly ($p < 0.05$) greater leaf P concentration than Merlin under control P, and significantly ($p < 0.05$) lower leaf P concentration than RGT Siroca following P addition. A significant ($p < 0.05$) P x phenological development interaction was observed, with P addition having a significantly ($p < 0.05$)

greater leaf P concentration at the R1 and R3 phase of phenological development by 41% and 25% respectively.

Stem P concentration significantly (p < 0.001) differs between phase of phenological developments, with concentration remaining constant from R1 to R3 (6% decrease), before decreasing from the R3 to R7 phase of phenological development. Phosphorus addition increased stem P concentration by 29%, with no significant (p = 0.930) differences between varieties. Pod P concentration significantly (p < 0.001) differs between phase of phenological development, with concentration remaining constant from R3 to R5 (4% increase) before decreasing by 84% to the R7 phase of phenological development. Root P concentration significantly (p < 0.001) differs between phase of phenological development, with concentration remaining constant from R1 to R3 (4% increase) before decreasing by 51% to R5 and remaining constant (8% decrease) to the R7 phase of phenological development. Phosphorus addition significantly (p < 0.001) increased root P concentration by 25%. Nodule P concentration significantly (p < 0.001) differs between phase of phenological developments, with a concentration decreasing by 37% between R1 and R3, and 22% between R3 and R5 before remaining constant (9% decrease) between R5 and R7. Phosphorus addition did not significantly (p = 0.125) influence nodule P concentration.

Phosphorus stock

A significant (p < 0.01) variety x phenological development interaction was observed in leaf P stocks (Table 5.5). Leaf P stocks increased significantly (p < 0.01) from R1 to R3 in both Merlin and RGT Siroca by 133% and 166% respectively (Figure 5.3.c). Leaf P stocks did not significantly differ between R3 and R5, Merlin then decreased significantly (p < 0.01) by 190% to R7 and RGT Siroca did not significantly differ (7% decrease). As a result, RGT Siroca leaf P stocks were significantly (p < 0.01) greater than Merlin at the R3 and R7 phase of phenological developments. Leaf P stocks were significantly (p < 0.01) greater following P fertiliser addition compared to control (43% increase).

Phosphorus stocks were significantly (p < 0.001) affected by phase of phenological development, with a significant (p < 0.001) increase observed between R1 and R3, and R3 and R5 by 151% and 45% respectively before decreasing significantly (p < 0.001) between the R5 and R7 phase of phenological development by 67%. Phosphorus addition significantly (p < 0.001) increased stem P stocks by 33%, and RGT Siroca had a significantly (p < 0.05) stem P stock than Merlin. Whilst not significant (p = 0.05), the R1 and R5 phase of phenological development appears to show differences between control and P fertiliser addition more than at the R3 and R7.

Pod P stocks were significantly (p < 0.001) affected by phase of phenological development, with a significant (p < 0.001) increase between R3 and R5 and a significant (p < 0.001) decrease between the R5 and R7 phase of phenological development. Root P stocks were significantly (p < 0.001) affected by phase of phenological development, with a significant (p < 0.001) increase of 97% between R1 and R3, a significant (p < 0.001) decrease of 46% between R3 and R5, with no significance between the R5 and R7 phase of phenological development. Phosphorus addition significantly (p < 0.01) increased root P stocks by 45% and RGT Siroca

had a significantly (p < 0.05) greater root P stock than Merlin. Nodule P stocks were significantly (p < 0.01) affected by phase of phenological development with a significant (p < 0.01) increase of 346% between R1 and R3, no significance between R3 and R5, and a significant (p < 0.01) increase of 24% between the R5 and R7 phase of phenological development. Phosphorus addition significantly (p < 0.001) increased nodule P stock by 25%, and RGT Siroca had a significantly (p < 0.01) greater P stock than Merlin.

5.3.3. Leaf macro- and micro-nutrient status

Potassium leaf tissue concentrations ranged from 1.64 – 32.39 mg g^{-1} (Table 5.6). A significant (p < 0.001) phenological development response was observed, with R7 having a significantly (p < 0.001) lower leaf K concentration that those at the at R1 – R5. Sulphur leaf tissue concentrations ranged from 1.46 – 8.30 mg g^{-1} (Table 5.6). A significant (p < 0.001) difference was observed at differing stages of phenological development, with R3 having a significantly (p < 0.001) greater leaf S concentration than R1 and R7 growth phases. Magnesium leaf tissue concentrations ranged from 1.46 – 9.59 mg g^{-1} (Table 5.6). No significant treatment responses were observed in leaf Mg concentration. Some significant differences in macro-nutrient concentrations occurred between the two varieties of soybean. RGT Siroca had a significantly (p < 0.01) greater leaf Ca and S concentration than Merlin. Merlin had a significantly (p < 0.01) greater leaf K concentration than RGT Siroca. No varietal differences were observed in leaf Mg concentration.

Boron leaf tissue concentrations ranged from 0.03 – 0.45 mg g^{-1} (Table 5.7). A significant (p < 0.01) P x variety interaction was observed, with Merlin showing no significant difference under differing P treatments, but RGT Siroca having increased leaf B concentration following P fertiliser addition. R7, leaf B concentration was significantly (p < 0.001) greater than R1- R5. Calcium leaf tissue concentrations ranged from 9.1 –46.8 mg g^{-1} (Table 5.7). A significant P x variety x phenological development three-way interaction was observed, with results of Bonferonni post hoc test showing the R7 stage of phenological development having a significantly (p < 0.001) greater leaf Ca concentration that those at the at R1 – R5. Within the control P treatments at R7, Ca concentration in RGT Siroca was significantly (p < 0.001) greater than Merlin and P addition of both varieties. Copper leaf tissue concentrations ranged from 0.004 – 0.043 mg g^{-1} (Table 5.7). A significant (p < 0.001) phenological development response was observed, with R1 having a significantly (p < 0.001) smaller leaf Cu concentration that those at the at R3 – R7. Iron leaf tissue concentrations ranged from 0.065 – 2.17mg g^{-1} (Table 4.7). A significant (p < 0.001) phenological development response was observed, with R7 having a significantly (p < 0.001) greater leaf Fe concentration that those at the at R3 and R1, and R1 having a significantly (p < 0.001) smaller leaf Fe concentration than R5 and R7. No significant differences were observed in Zn leaf tissue concentrations, with Zn concentrations ranging from 0.01 – 0.24 mg g^{-1} (Table 5.7).

Table 5.6. Leaf tissue macro-nutrient (Calcium, Potassium, Magnesium and Sulphur) concentration (mg g^{-1}) of two varieties of soybean (Merlin and RGT Siroca) grown under two phosphorus (P) treatments (control and P fertiliser addition). Individual plants in pots were harvested at four phases of phenological development (R1, beginning of flowering; R3, beginning of pod formation; R5, beginning of pod filling; and R7, beginning of full maturity). Data are means ± SE of 6 replicates. Three-way ANOVA *p* values are displayed. Green shading represents nutrient deficiency (Sale and Campbell, 1986).

Source of Variation			Macro-nutrient concentration (mg g^{-1})		
Phase of phenological development	Variety	Phosphorus	Potassium	Magnesium	Sulphur
R1	Merlin	Control	13.44 ± 0.73	3.49 ± 0.29	1.74 ± 0.13
R1	Merlin	P addition	14.98 ± 0.97	3.42 ± 0.22	1.65 ± 0.06
R1	RGT Siroca	Control	16.95 ± 1.23	3.14 ± 0.21	1.99 ± 0.09
R1	RGT Siroca	P addition	16.79 ± 0.82	3.53 ± 0.23	1.97 ± 0.10
R3	Merlin	Control	19.61 ± 2.13	4.20 ± 0.37	2.69 ± 0.38
R3	Merlin	P addition	19.39 ± 2.20	3.51 ± 0.29	1.90 ± 0.22
R3	RGT Siroca	Control	15.22 ± 3.19	3.60 ± 0.37	2.31 ± 0.47
R3	RGT Siroca	P addition	19.34 ± 4.45	5.84 ± 2.33	2.94 ± 0.55
R5	Merlin	Control	17.80 ± 0.56	3.32 ± 0.28	1.97 ± 0.09
R5	Merlin	P addition	17.72 ± 2.30	3.35 ± 0.60	2.07 ± 0.23
R5	RGT Siroca	Control	16.39 ± 0.70	3.13 ± 0.18	1.93 ± 0.17
R5	RGT Siroca	P addition	18.17 ± 2.06	5.51 ± 2.30	1.94 ± 0.17
R7	Merlin	Control	4.21 ± 1.35	3.57 ± 0.49	1.41 ± 0.16
R7	Merlin	P addition	3.49 ± 1.32	5.49 ± 0.96	1.34 ± 0.12
R7	RGT Siroca	Control	6.47 ± 2.45	5.55 ± 0.97	2.07 ± 0.25
R7	RGT Siroca	P addition	4.90 ± 0.09	5.82 ± 1.14	1.70 ± 0.24
		Phosphorus (P)	0.694	0.174	0.564
		Variety (V)	<0.01	0.063	< 0.05
		Phase of phenological development (GP)	< 0.001	0.071	< 0.001
		P *V	0.497	0.214	0.390
		P * GP	0.458	0.299	0.820
		V * GP	0.579	0.265	0.133
		P * V * GP	0.073	0.692	0.082

Table 5.7. Leaf tissue micro-nutrient (boron, copper, iron and zinc) concentration (mg g^{-1}) of two varieties of soybean (Merlin and RGT Siroca) grown under two phosphorus (P) treatments (control and P fertiliser addition). Individual plants in pots were harvested at four phases of phenological development (R1, beginning of flowering; R3, beginning of pod formation; R5, beginning of pod filling; and R7, beginning of full maturity). Data are means ± SE of 6 replicates. Three-way ANOVA *p* values are displayed. Pink shading represents nutrient toxicity (Burton et al., 2000; Pawlowski et al., 2019).

Source of Variation			Micro-nutrient concentration (mg kg^{-1})				
Phase of phenological development	**Variety**	**Phosphorus**	**Boron**	**Copper**	**Calcium**	**Iron**	**Zinc**
R1	Merlin	Control	0.03 ± 0.00	0.01 ± 0.00	14.41 ± 1.00	0.1 ± 0.01	0.06 ± 0.04
R1	Merlin	P addition	0.03 ± 0.00	0.01 ± 0.00	13.60 ± 1.36	0.1 ± 0.01	0.05 ± 0.00
R1	RGT Siroca	Control	0.03 ± 0.00	0.01 ± 0.00	15.52 ± 1.03	0.09 ± 0.01	0.05 ± 0.00
R1	RGT Siroca	P addition	0.03 ± 0.00	0.01 ± 0.00	19.55 ± 1.76	0.13 ± 0.02	0.05 ± 0.00
R3	Merlin	Control	0.04 ± 0.01	0.02 ± 0.00	19.66 ± 1.80	0.14 ± 0.01	0.08 ± 0.00
R3	Merlin	P addition	0.06 ± 0.02	0.01 ± 0.00	18.23 ± 2.77	0.16 ± 0.03	0.06 ± 0.01
R3	RGT Siroca	Control	0.06 ± 0.01	0.02 ± 0.00	24.88 ± 2.03	0.21 ± 0.03	0.06 ± 0.01
R3	RGT Siroca	P addition	0.05 ± 0.01	0.02 ± 0.00	16.08 ± 1.95	0.22 ± 0.06	0.06 ± 0.01
R5	Merlin	Control	0.08 ± 0.00	0.02 ± 0.00	17.65 ± 1.99	0.26 ± 0.05	0.05 ± 0.01
R5	Merlin	P addition	0.08 ± 0.00	0.02 ± 0.00	19.06 ± 0.89	0.34 ± 0.09	0.05 ± 0.01
R5	RGT Siroca	Control	0.1 ± 0.01	0.02 ±0.00	20.43 ± 1.04	0.23 ± 0.03	0.07 ± 0.01
R5	RGT Siroca	P addition	0.07 ± 0.02	0.02 ± 0.00	18.50 ± 1.85	0.23 ± 0.03	0.07 ± 0.01
R7	Merlin	Control	0.27 ± 0.06	0.02 ± 0.00	25.96 ± 4.47	0.40 ± 0.33	0.05 ± 0.01
R7	Merlin	P addition	0.43 ± 0.12	0.02 ± 0.01	37.62 ± 4.65	0.49 ± 0.39	0.04 ± 0.01
R7	RGT Siroca	Control	0. 28 ± 0.07	0.02 ± 0.01	39.50 ± 1.78	0.56 ± 0.31	0.05 ± 0.00
R7	RGT Siroca	P addition	0. 45 ± 0.04	0.01 ± 0.00	34.63 ± 3.22	0.46 ± 0.06	0.07 ± 0.03
		Phosphorus (P)	< 0.05	0.396	0.509	0.803	0.509
		Variety (V)	< 0.05	0.936	< 0.01	0.905	< 0.01
		Phase of phenological development (GP)	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
		P *V	< 0.05	0.604	0.06	0.400	0.06
		P * GP	0.474	0.745	0.084	0.684	0.084
		V * GP	0.295	0.064	0.429	0.529	0.429
		P * V * GP	0.103	0.457	< 0.01	0.224	< 0.01

5.4. Discussion

5.4.1. Varietal differences in plant phosphorus uptake and concentration

RGT Siroca had significantly ($p < 0.01$) greater plant P uptake across the growth cycle, with increased whole plant P stocks at maturity, however no significant differences ($p = 0.341$) were observed between the varieties in whole plant P concentrations (Figure 5.1). The increase in plant P uptake is likely to be associated with the significantly ($p < 0.001$) greater plant biomass observed in RGT Siroca compared to Merlin (Table 5.1). Whilst root morphology was not quantified in this study, RGT Siroca was found to have significantly ($p < 0.001$) greater root biomass, this increase in plant P uptake may be associated with RGT Siroca having a more beneficial root system to explore greater volumes of soil for P. Several beneficial morphological traits leading to a more extensive or efficient root system (including greater length, volume and specific root length and the presence of root hairs) enable improved access to soil P stores through occupying a greater soil volume(Brown et al., 2013; Kumar et al., 2019; Liang et al., 2010; Wang et al., 2010). The advantages of this have been demonstrated in other legumes, such as bean (*Phaseolus vulgaris*) where genotypes with highly branched, actively growing root systems have been shown to more P efficient (Lynch et al., 2001). The potential mechanisms of RGT Siroca to acquire more P may also contribute to the increased sensitivity of Merlin to P fertiliser addition, with Merlin whole plant P stocks increasing by 92.8% compared to 33.8% in RGT Siroca. Plant P uptake under control P treatments, whilst not significant, is lower in Merlin than RGT Siroca, suggesting reduced capability to mine P under low P conditions. The increased uptake may also have arisen due to RGT Siroca having a greater growth potential, observed through significantly ($p < 0.01$) greater net photosynthesis observed in chapter 4 (Figure 4.3.d.), and therefore able to increase biomass production and hence requiring more P.

5.4.2. Phosphorus stock remobilisation and partitioning throughout the growth cycle

All plant organs increased or maintained biomass as growth progressed, except for leaf tissue which declined significantly ($p < 0.001$) between the R5 and R7 phase of phenological development because of leaf senescence (Figure 5.3.a). Phosphorus concentration did not follow the same pattern as biomass accumulation; stem leaf and pod all had a significant ($p < 0.001$) decline in concentration between the R5 and R7 phase (Figure 5.3.b). The combined dynamics of biomass allocation and variation in P concentrations led to significant differences in P stocks amongst organs (Figure 5.3.c).

Root P stocks peaked at R3 before reducing as development progressed. At R3 P demand is high with P uptake found to increase following initiation of reproductive growth (Bender et al., 2015; Gaspar et al., 2017; Hanway and Weber, 1971). Increased root P stocks aid in improving P uptake through the provision of ATP, providing energy for root cell metabolism and growth, and the synthesis and activation of enzymes involved in nutrient acquisition and transport (George et al., 2011; Khan et al., 2023; Shen et al., 2006).

Nodule P stocks peaked at R5, this corresponds with the observed peak in N_2 fixation (Figure 4.1.a, (Ciampitti et al., 2021)). Nodule P concentration was not significantly influenced by P fertiliser addition, with nodule biomass and P stocks following similar patterns of increase throughout the growth stages under different varieties and treatments. This corresponds with other studies that have suggested that legume crops can adapt to low P concentration through the maintenance of P homeostasis in nodules (Liu et al., 2018; Sulieman and Phan Tran, 2105). The upregulation of expression of genes implicated in direct P uptake by nodules, transporter genes to control the transport of P between host roots and nodules, and genes relative to P recycling from organic substances and recycling inorganic P from other tissues is thought to contribute to the mechanisms maintaining nodule P concentration under low P conditions (Cao et al., 2021; Chen et al., 2019; Lu et al., 2020; Zhong et al., 2023). Leguminous crops have also been found to adapt to low P concentrations through increasing phytase and phosphatase activity in nodules (Araújo et al., 2008; Mandri et al., 2012). These mechanisms to maintain nodule P status under low P conditions helps maintain nodule functioning and N_2 fixation under low P conditions.

Varieties differed primarily in leaf P stock patterns, with Merlin peaking at R5 before reducing towards R7 and RGT Siroca peaking at R3 and stabilising to the R7 phase of phenological development. The increase in P concentration and resultant P stocks of RGT Siroca at R3 suggests an increase in P available for photosynthetic processes, promoting the observed increase in net photosynthesis and plant growth (Figure 4.3.d; Table 5.1). Leaf photosynthetic capacity is limited by P through mechanisms such as the reduction in ribulose-1,5-biphosphate regeneration, carboxylation activity, light use efficiency and stomatal conductance (CAMPBELL and SAGE, 2006; Singh et al., 2018; Singh et al., 2019). The maintained leaf P stock in RGT Siroca at R5 was not however reflected in increased net photosynthesis at this growth phase, with no significant differences observed between the varieties at R5 (Figure 4.3.d).

At R7, Merlin allocated more biomass to the seed than RGT Siroca (40.7% of total biomass compared with 35.9%), alongside Merlin reducing allocation to both the pod and the leaf compared with RGT Siroca (Figure 5.2.b.). Merlin had a significantly ($p < 0.001$) lower leaf biomass at the R7 phase of phenological development compared with RGT Siroca, with increased levels of leaf senescence observed, consistent with Merlin being an earlier maturity variety. A similar relationship was observed with P partitioning, with Merlin having increased P allocation to the seed (81% of total P compared with 72% in RGT Siroca) (Figure 5.2.a.). Efficient nutrient recycling as leaf senescence occurs requires optimising the highly ordered multistep process of biochemical, physiological and metabolic alteration, chlorophyll degradation and cessation of photosynthetic activity (Aloryi et al., 2023; Munné-Bosch and Alegre, 2004). Whilst leaf P concentrations can decrease by nearly 80%, the mechanisms of recycling and remobilisation are less understood (Smith et al., 2017). Phosphorus is released during the catabolism of P containing biomolecules (including RNA, acid phosphatases and phospholipids) which are then exported for transport to sink tissues via orthophosphate transporters (Stigter et al., 2015). These results suggest Merlin is more efficient at reallocating resources away from leaves to seed as leaf senescence occurs.

5.4.3. Phosphorus harvest index and seed phosphorus concentration

The translocation of P to the seed tends to be more intense than that of photosynthates, with proportionally higher PHI than HI (Araújo et al., 2003; Veneklaas et al., 2012), as observed in this study. The maintenance of leaf P in RGT Siroca can improve late canopy photosynthesis, a potential strategy to extend the seed filling period and increase grain yield (Lynch and Rodriguez, 1994; Phillips et al., 1984). Whilst late season photosynthesis was not quantified, RGT Siroca had significantly ($p < 0.01$) greater thousand seed weight, suggesting bigger seeds resulting from improved seed filling (Table 4.2). However, Merlin had both an increased PHI and HI (Figure 5.1), suggesting whilst having reduced growth and P uptake, the variety is more efficient at utilising and reallocating resources (including P) to reproductive organs with increased biomass and P partitioned to seed (Figure 5.2) and increased seed number per pod (Table 4.2).

Observed seed P concentrations were within the ranges previously reported (e.g. (King et al., 2012; Krueger et al., 2013)) and as expected, seed P concentrations was significantly greater ($p < 0.001$) following P fertiliser addition. Whilst P is required within the seed because of its role in the production of seed and the germination success of the seed in future growth seasons, excessive seed P stores can be detrimental to seed quality. For example, Linoleic acid, a polyunsaturated fatty acid that cannot be synthesised by the body, concentrations have been found to decrease with increasing P fertilisation (Carroll, 1986; Krueger et al., 2013). The influence of seed P concentration on seed oil and protein concentrations are inconclusive, with P application being found to increase oil and protein concentrations (Abbasi et al., 2012; Taliman et al., 2019), or have no correlation with protein concentration and decrease oil concentration (Bethlenfalvay et al., 1997).

The majority of P stored in the seed is in the form of phytate, a salt comprising of negatively charged phytic acid and cations including K^+ and Mg^{2+}, comprising approximately 78% of seed P and 1-2% of seed composition (King et al., 2012; Reddy et al., 1982). Reduced seed phytate concentration can negatively affect seed vigour, with phytate being hydrolysed by endogenous phytase, releasing stored P and minerals to be utilised during seed development (Raboy, 2009). However, phytate cannot be digested by humans and other monogastric animals, with phytate restricting the bioavailability of iron and zinc that can be consumed (White and Broadley, 2009). As a result, high phytate concentration in the seed is often considered an undesirable trait for soybean seed quality. Indigested seed phytate results in the release of additional P in manure (Lott et al., 2011). This additional P in manure contributes to the problem of agricultural pollution in waterways, quickening the onset of eutrophication (Brinch-Pedersen et al., 2002; Wiggins et al., 2018). When P remains in crop residue, such as senescing leaves, rather than being remobilised to the seed as indigestible phytate, this crop residue can be reincorporated into the soil, reducing the need for P fertiliser addition.

5.4.4. Leaf nutrient status

Calcium leaf concentration increased significantly ($p < 0.001$) at R7 (Table 5.6). Unlike other nutrients, Ca is considered immobile (White and Broadley, 2009; White, 2012). As a result of the combined immobility of Ca and the remobilisation of other micro- and macro-nutrients, the increased concentration of Ca at R7 is not unexpected. It has been reported that the uptake of Ca and accumulation in leaf tissue above certain thresholds act as a mediator of leaf senescence, consistent with the observed colour change and leaf abscission (Ma et al., 2010). Potassium leaf concentration decreased significantly ($p < 0.001$) at R7 (Table 5.6). Leaf K concentrations ≤ 15 g K kg^{-1} is considered deficient at the R1 – R2 growth phase and whilst not quantified this threshold is thought to decrease as growth continues (Borges and Mallarino, 2000; Grove et al., 1987; Krueger et al., 2013; Slaton et al., 2021). The decline in leaf K concentration as soybean growth continues agrees with previous research and is attributed to a combination of dilution arising through increased dry matter production and the translocation of K to the seed, with increased seed K concentration being correlated with seed quality (Hanway and Weber, 1971; Jeffers et al., 1982; Krueger et al., 2013) Sulphur concentrations within this study were found to be within critical thresholds previously reported (Divito et al., 2015). Whilst S is remobilised in the plant to the seed, it is considered to have low mobility in plants, with S mobilisation from vegetative to reproductive plant tissue is less efficient than N (de Borja Reis et al., 2021; Naeve and Shibles, 2005; Sexton et al., 2002). Sulphur-containing amino acids, cysteine and methionine are found in soybean seed and play a vital role in human health and nutrition (Krishnan et al., 2012; Y and H, 2013). As a result, the remobilisation of S from the leaf to the seed is imperative in improving soybean seed quality. No significant treatment responses were observed in leaf Mg concentration; this is consistent with work by Bender et al. (2015), where limited differences in leaf Mg accumulation and partitioning were observed. RGT Siroca had a significantly ($p < 0.01$) greater leaf Ca and S concentration than Merlin. Merlin had a significantly ($p < 0.01$) greater leaf K concentration than RGT Siroca. Potassium efficiency differs amongst soybean varieties, with higher harvest index (HI) found in high-efficiency varieties (Liu et al., 2019) suggesting that the increased leaf K concentration of Merlin may contribute to the increased HI observed (Figure 4.5.b).

Boron leaf tissue concentration of RGT Siroca was significantly ($p < 0.01$) greater following P fertiliser addition, with no significant difference observed in Merlin. The increase leaf B concentration of RGT Siroca is within the optimal range for soybean growth, with soybean being reported to be relatively insensitive to B deficiency (Martens and Westermann, 1991; Ross et al., 2006; Touchton and Boswell, 1975). The enhanced plant metabolic activity associated with P fertiliser addition may have increased demand for B, which lead to the increased leaf B concentration(Shorrocks and Shorrocks, 1997). At the R7 growth phase, leaf B concentration was significantly ($p < 0.001$) greater than the R1- R5 growth phase. Boron is immobile in soybean, and as a result it is not remobilised from the leaves to the seed as leaf senescence occurs (White, 2012). The combination of the immobility of B and the remobilisation of other micro- and macro-nutrients may have contributed to the toxic levels of B exhibited in this study.

5.5. Conclusion

Whilst P uptake and resultant plant and seed P concentration increases following P fertiliser addition, P remobilisation to the seed and hence PHI of soybean are not significantly influenced by P fertiliser treatment. Results showed varietal differences in P uptake, biomass and P remobilisation strategies – whilst RGT Siroca exhibited 43.9% greater P uptake and accumulation (likely attributed to its larger biomass and potentially more efficient root system), Merlin showed superior P and biomass remobilisation efficiency, reflected in its higher PHI and HI. Merlin successfully allocated more P and biomass to seeds, resulting in comparable seed yield (17.8 g plant^{-1}) to RGT Siroca (17.1 g plant^{-1}), despite the smaller plant size. These results confirm the hypothesis that Merlin has more efficient remobilisation and partitioning of P during reproductive growth. The observed seed yield advantage of Merlin in response to phosphorus addition, identified in Chapter 4, is underpinned by its efficient nutrient utilisation and remobilisation strategies. Future research should focus on unravelling the physiological and genetic mechanisms governing P remobilisation, with particular focus on leaf senescence dynamics and their influence on seed quantity and quality. Merlin's earlier senescence and efficient phosphorus redistribution to seeds appear to maximize resource use for reproductive success. However, the implications of increased redistribution of P to seed may increase seed phytate concentration that can be detrimental for both seed quality and the environment. Breeding and management strategies must balance seed yield improvement with P use efficiency, ensuring that increases in seed P do not compromise seed quality or environmental sustainability.

6. General Discussion

This chapter revisits the aims, objectives and hypotheses of this thesis and provides a general discussion presenting the wider findings from across the thesis in context with suggestions for future research.

6.1. Summary of aims and key questions

Phosphorus (P) fertiliser use within soybean production has yet to be optimised, with improved understanding of soybean response to P fertiliser addition required to inform the sustainable use of P fertiliser. Sufficient soil P supplies are required to improve yields, however, solutions to the difficulties in managing P fertiliser to increase production whilst minimising environmental costs long debated within the scientific literature have not been achieved. This research aimed to bridge the gap between mechanistic understanding of processes following P fertiliser addition, and field scale P fertiliser recommendations. Through analysis of various experiments at differing scales, from pot trial to field trials and the completion of a global meta-analysis, this thesis sought to test the hypotheses that soybean crop growth is P limited, and that improving P fertiliser use could optimise nitrogen (N_2) fixation processes and increase yield. To test this hypothesis this thesis answered the following questions:

1. How does phosphorus fertiliser addition improve nitrogen fixation?
2. What is driving the increase in soybean yield following phosphorus fertiliser addition?
3. Does the relationship between phosphorus fertiliser addition and soybean yield differ at the global scale?

6.2. How does phosphorus fertiliser addition improve nitrogen fixation?

Whilst P is widely recognised to play a key role in N_2 fixation processes, gaining focus in research over recent years, the underlying responses driving increased N_2 fixation remain elusive. The number of studies examining N_2 fixation and nodulation in soil systems is much smaller than that of hydroponic studies, with chapter two revealing only seven studies in a soil system that fit the criteria of the meta-analysis. Through multiple studies, this thesis illustrated the interaction between P fertiliser addition and inoculation, with P fertiliser being shown to increase N_2 fixation. The first question posed in this thesis was driven by testing two hypothesised pathways of improved N_2 fixation:

1. Phosphorus fertiliser addition increased nodule formation through increased resource accumulation and allocation to improve N_2 fixation
2. Phosphorus fertiliser addition increased nodule function to improve N_2 fixation

This thesis found that beneficial nodule traits increased following P fertiliser addition (Table 10.2, Figure 4.2a – Figure 4.2.d), with chapter four showing a significant correlation between nodule dry weight or nodule number and shoot N (Figure 4.4), and as a result hypothesis one has been accepted. In contrast, leghaemoglobin concentration whilst showing a trend of

increase following P fertiliser addition (particularly in earlier stages of phenological development), shows recovery at later stages with control treatments having increased concentration (Figure 4.2.e). Alongside, the correlation between nodule traits and shoot N not being significantly influence by P fertiliser treatment (Table 4.5) suggest P addition increases nodule formation but does not influence N_2 efficiency per unit of nodule. As a result, hypothesis two was rejected. The understanding of the role of P fertiliser addition on soybean N_2 fixation generated in this thesis, and possible mechanisms have been summarised in Figure 6.1.

An increase in nodule number following P fertiliser addition was observed (Table 10.2, Figure 4.2.a), corresponding with other studies in the literature, because of an increase in nodule formation (e.g. (Jin et al., 2022; Qiao et al., 2007a)). Alongside the increase in nodule number, an increase in nodule mass and an increase in specific nodule mass (nodule mass per unit of root mass) was observed following P fertiliser addition (Figure 4.2). This suggests an increased allocation of resources to the nodules, confirming what has been observed in studies such as (Li et al., 2021b; Sulieman et al., 2022). Interestingly, in the second pot trial completed in chapter 3 where nodules were classified by size (small <2 mm diameter and large ≥ 2mm diameter), a difference between the two varieties was observed (Table 10.2). Despite both varieties showing an increase in nodule mass, only the Merlin variety had an increase in larger nodules following P fertiliser addition RGT Siroca did not show any difference in nodule size. The larger nodule size observed in the Merlin variety following P addition did result in higher N_2 fixation. The two varieties differed in their N_2 fixation under the differing environmental and experimental conditions (Table 6.1), corresponding with findings from existing literature (Keyser and Li, 1992; Schipanski et al., 2010). Varieties also had different N_2 fixation capacities at different stages of phenological development (Figure 4.1), with Merlin characterised as an early fixer and RGT Siroca as a later fixer(Hamawaki and Kantartzi, 2018).

Table 6.1. Average Relative abundance of ureide (RAU %) of two varieties of soybean (Merlin and RGT Siroca) under differing environmental conditions and experimental systems. Variations in phosphorus (P) fertiliser treatment, with soil available P concentration prior to planting, harvest point and experimental condition are listed with average Relative abundance of ureide (%) of Merlin and RGT Siroca.

Experimental system	Harvest point	P Treatment (Soil available P concentration prior to planting)	RAU %	
			Merlin	RGT Siroca
Field (Chapter Three)	R2	NA (8 mg P kg^{-1})	16.7	17.0
Controlled environment 4 l pot size (Appendix C)	R2	Control (6 mg P kg^{-1})	52.3	66.6
		P addition (23 mg P kg^{-1})	88.9	92.2
Controlled environment 0.75 l pot size (Chapter Three)	R1	Control (6 mg P kg^{-1})	69.0	-
		P addition (23 mg P kg^{-1})	73.0	-
Controlled environment 4 l pot size (Chapter Four and Five)	R1	Control (6 mg P kg^{-1})	32.2	8.9
		P addition (23 mg P kg^{-1})	62.9	36.8
	R3	Control (6 mg P kg^{-1})	86.6	90.4
		P addition (23 mg P kg^{-1})	79.8	87.9
	R5	Control (6 mg P kg^{-1})	94.3	95.4
		P addition (23 mg P kg^{-1})	97.4	96.3
	R7	Control (6 mg P kg^{-1})	19.4	64.1
		P addition (23 mg P kg^{-1})	22.7	56.3

Through sampling at four stages of development the temporal pattern of N_2 fixation across the growth cycle of soybean is shown to respond more significantly to P addition at the vegetative stage, before the apparent recovery under control conditions, with N_2 fixation peaking at the beginning of pod formation (R5) before decreasing as plants approach maturity. The recovery of leghaemoglobin concentration and relative ureide concentration (RAU) is hypothesised to be due to regulatory mechanisms to mitigate stressors such as P stress in the control treatments. In chapter five one such regulatory mechanism explored was the regulation of P concentration within the nodule. The increase in P concentration following P addition across both varieties and all phenological stages measured was 1.6%, lower than the increase in other plant organs and whole plant increase (Table 6.2.). The partitioning of P stock (%) to the nodules was found not to significantly differ between P treatments (p = 0.750) or varieties (p = 0.995) From these results it can be inferred that whilst P fertiliser addition increases nodule P concentration, the P homeostasis of nodules (particularly under low P conditions) aid in the maintenance of N_2 fixation.

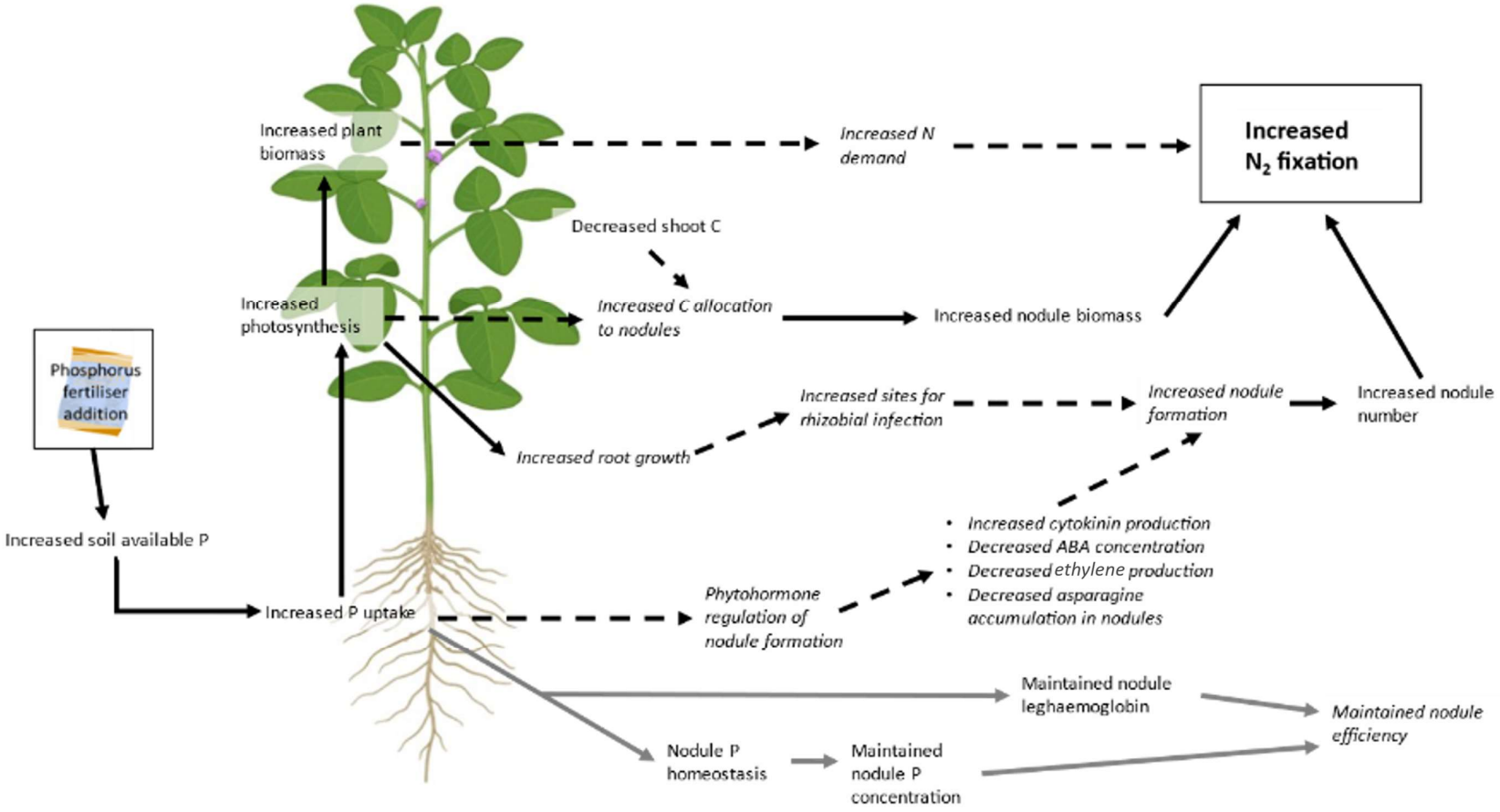
Table 6.2. Percentage increase in phosphorus (P) concentration in P fertiliser addition compared to control fertiliser treatment. Calculated based on mean P concentration in both varieties at the four measured phenological stages (R1, beginning of flowering; R3, beginning of pod formation; R5, beginning of pod filling; and R7, beginning of full maturity), in a controlled environment study, with 6 replicates of each variety at each measured phenological stage.

Plant Organ	P concentration increase (%)
Whole plant	21.6
Nodule	1.6
Root	33.9
Leaf	33.5
Stem	45.4
Pod	58.4
Seed	25.5

To fully understand the process of N_2 fixation and apply findings more robustly across differing scales improvements need to be made to better quantify fixation processes. The difficulties associated with measuring N_2 fixation in soybean, particularly the newer, European varieties studied in this thesis was discussed in chapter three and appendix B. The relative abundance of ureide (RAU) method, which was used throughout the thesis, because of constraints associated with the use of isotopic analysis, has some limitations. Measured RAU can be affected by environmental conditions, and differences amongst varieties without corresponding changes in N_2 fixation being observed (Purcell et al., 2004). This was also observed in chapter three, with no significant correlation being found between the RAU method and $\delta^{15}N$ natural abundance methods. Difficulties arise in calculating mass of N_2 derived from fixation (Ndfa); for example, whilst P addition was proven to increase RAU%, shoot N concentration and shoot N mass in chapter four the effect of P addition on the mass of Ndfa cannot be calculated as the linear models proposed by Herridge and Peoples (1990) overestimated Ndfa, giving percentages greater than 100%. As a result, one area suggested for further research is the improved facilitation of methods to measure N_2 fixation and ensuring these methods can be applied to all environments, genotypes and management

conditions. If resources and facilities were more readily available, it would have been beneficial to use $\delta^{15}N$ natural abundance methods throughout this thesis to quantify N_2 fixation, however this was not possible due to the higher cost and requirements of additional growth space for the growth of a suitable reference crop.

Figure 6.1. Conceptual diagram summarising the processes involved in increased nitrogen (N_2 fixation) following phosphorus (P) fertiliser addition in soybean. Solid arrows indicate processes established in this thesis, with black arrows representing resource-driven response and grey arrows represent stability-driven homeostatic processes. Dotted arrows represent possible mechanisms.



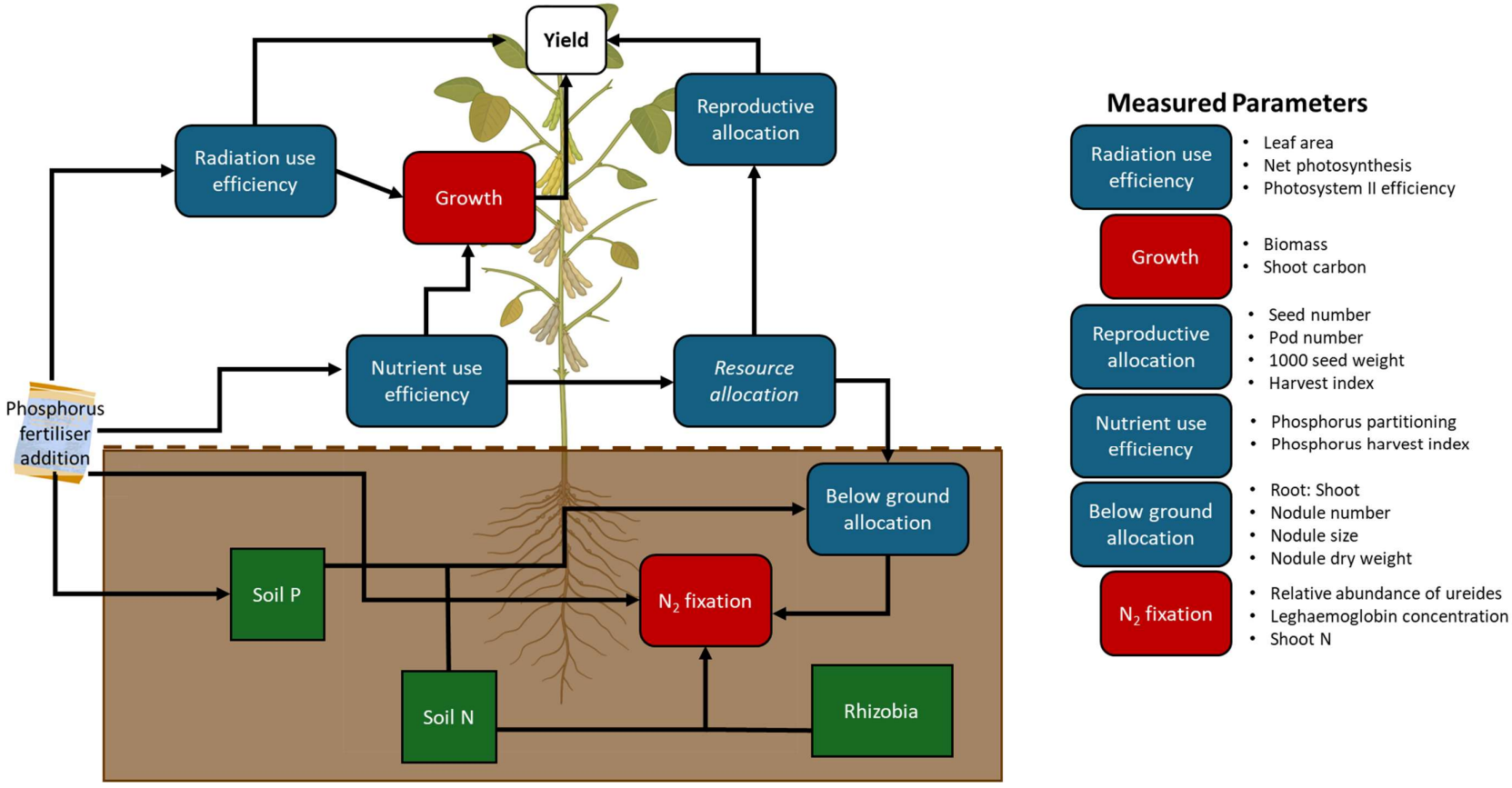
6.3. What is driving the increase in soybean yield following phosphorus fertiliser addition?

The fundamental principles of plant growth and plant resource use metrics at the whole plant level examined in this thesis have been summarised in a conceptual diagram (Figure 6.2). In Chapter two the mean seed yield increase following P fertiliser addition was approximately 21%, with mean fertiliser application rate being 47 kg P ha^{-1}, and in Chapter four the observed seed yield increase across both varieties was 25% increase following a fertiliser application rate of 60 kg P ha^{-1}. Soybean seed yield is thought to be determined by several processes that in turn can be influenced by environmental, management and genotypic differences.

The determination of pod number is influenced by the combination of node number (in turn influenced by plant height, branching, internode length and variety), canopy photosynthesis and plant growth until pod setting, with these processes being influenced by P fertiliser addition (Egli, 2013; Egli and Bruening, 2006; Egli and Bruening, 2007). Thousand seed weight showed a trend of increase following P fertiliser addition (7.4% increase, $p = 0.058$) compared to the control (Table 4.6). Similar results have been observed in other legumes (Ouedraogo et al., 2024). Conversely, seed number per pod was not found to be significantly different following P fertiliser addition (Table 4.6) suggesting the mechanisms involved in seed setting are not driven by P fertiliser addition.

Alongside the increased yield parameters because of increased plant growth due to P fertiliser addition, there is also an increase in allocation to the seed with harvest index (HI) increasing by approximately 10% following P fertiliser addition and pod harvest index (podHI) having a trend of increase ($p = 0.66$) of approximately 3% following P fertiliser addition (Figure 4.5b and Table 4.6). Results of Chapter five provides empirical evidence of both increased P (79% of total P allocated to the seed under P addition compared with 75% under control treatment) and biomass allocation (41% of total biomass allocated to the seed under P addition compared with 37% under control treatment) to the seed following P fertiliser addition (Figure 5.2). The extent to which P is translocated to grains is often overlooked when considering P use efficiency, with contrasting evidence as to if increased grain P is a positive trait (Wang et al., 2016). It is hypothesised that because of increased shoot C from photosynthesis processes and increased shoot N from N_2 fixation processes following P addition, there would also be an increase in N and C remobilisation to the seed, however this needs to be confirmed through future experimental work.

Figure 6.2. A conceptual model of soybean yield response following phosphorus (P) fertiliser addition. Green boxes represent soil parameters, red boxes represent key processes, and blue boxes represent physiological parameters that may be influenced by P fertiliser. Measured parameters from this thesis are shown on the right side of the figure**.**



6.4. Does the relationship between phosphorus fertiliser addition and soybean yield differ at the global scale?

In chapter one, the reworking of data provided by (McDowell et al., 2023) estimated large proportions of soils in the top four soybean producers are P limited for soybean. Through the upscaling of research conducted in this thesis in combination with the reworking of data from chapter one, the economic costs of increased P fertiliser application and estimated economic benefits of yield increase if soil P status in the top four soybean producing countries was achieved has been summarised in Table 6.3. If estimated yield increases close to what has been observed in this thesis could be achieved through additional P fertiliser application, large economic benefits (estimated to be approximately $30 billion, not accounting for other economic costs associated with achieving increased yields) could be realised. If yield increases of 20% could be replicated at the field scale of production it would make a large contribution to closing existing yield gaps estimated to be approximately 35% (Di Mauro et al., 2018; Merlos et al., 2015; Sentelhas et al., 2015). It must be noted however, that this yield increase was achieved in a sandy soil under controlled environmental conditions, so the likelihood of achieving this increase at the field scale whilst only altering P fertiliser practices is very low.

The results of Chapter two, provided valuable insight into the implications of environment and management practices on the relationship between P fertiliser addition and seed yield, highlighting how these practices and conditions influence soybean response. Despite a lack of reporting of environmental conditions making analysis difficult, the results of chapter two highlighted the importance of soil chemistry (with pH explaining 36% of the variance observed in seed yield response) and climatic variation (53% of the variance observed) in influencing the efficiency of P fertiliser addition. Following this, the controlled environment studies of this thesis used only one or two varieties of soybean primarily under one set of environmental and management conditions. This work contradicts the discussion of Chapter two in which the importance of studying response to P fertiliser addition under a range of environmental and management practices was discussed. However, unfortunately due to the constraints associated with cost, time and labour resource this could not be implemented.

Table 6.3. Summary of estimated phosphorus (P) fertiliser requirements, economic costs, yield increases and economic benefit of applying 50 kg P ha to P limited soils in the top four soybean producing countries. Current yield, production and production calculated using FAO statistical data for 2023 (FAOSTAT, 2023), predicted P limited soils and area calculated using data provided by McDowell et al. (2023).

	Current production level			**Predicted soil P**		**Estimated costs and benefits of additional P fertiliser**					
Country	Yield (kg ha^{-1})	Production area (Mha)	Production (Mt)	Phosphorus limited[a] soils (%)	Phosphorus limited[a] area (Mha)	Fertiliser requirements (Kt P)[b]	Economic cost of fertiliser (million U.S.$)[c]	Improved yield (kg ha^{-1})	Total production (Mt)	Production increase (Mt)	Economic benefit (billion U.S.$)[d]
Brazil	3423.0	44.4	152.1	97.1	43.1	22.2	10.9	4107.6	182.3774	30.3	18.1
U.S.	3398.7	33.3	113.3	91.3	30.4	16.7	8.2	4078.44	135.8121	22.5	13.3
Argentina	1744.5	14.4	25.0	59.2	8.5	7.2	3.5	2093.4	30.14496	5.1	3.1
China	1952.6	10.0	19.5	15.5	1.5	5.0	2.5	2343.12	23.4312	3.9	2.4

[a] ≤ 9 mg P kg^{-1} Olsen's available phosphorus

[b] Estimated P fertiliser requirements of 50 kg P ha^{-1} giving 20% yield improvement, based on results collected in this thesis

[c] Estimated based on economic cost of triple superphosphate (TSP) November 2024 (491 U.S.$ tonne^{-1}, World Bank (2024))

[d] Economic benefit of 20% yield improvement (cost of increased production minus cost of fertiliser requirements, estimated based on average market price for soybean worldwide in 2023 (598 U.S.$ tonne^{-1}, World Bank (2024))

6.5. Directions for future research

Whilst the works of this thesis corroborate current understanding that if a soil is P limited, the addition of P fertiliser will increase seed yield and N_2 fixation, enhancing knowledge of the processes involved in achieving this increase. However, it has also broader questions for future research:

Does phosphorus fertiliser addition alter seed quality*?* The results of chapter five suggest seed P concentration also increases following P fertiliser addition. Current research is inconclusive on the influence of seed P concentration on seed oil and protein concentrations (Abbasi et al., 2012; Bethlenfalvay et al., 1997; Taliman et al., 2019). As a result, future research should focus on understanding if the increased yield achieved by P fertiliser addition is at the detriment of quality, as this will have confounding effects on food security concerns. If the additional seed yield achieved by P fertiliser addition is poorer quality, future research should focus on strategies to mitigate this, such as breeding low phytate genotypes or the addition of other management strategies to improve seed quality.

How does phosphorus stress influence soybean production in combination with other environmental and management stressors? The focus of this thesis has been on improving P fertiliser use to negate P stress response in soybean, with results of chapter two showing how other environmental and management conditions alter the relationship between P fertiliser addition and yield. However, the experimental studies of this thesis (except for the incorporation of low light stress in Appendix C) have examined P stress in isolation. Several environmental and management stressors limit soybean production, including, but not limited to, sub-optimal soil nutritional status, drought, water deficit, heat stress, cold stress, light availability and lack of successful rhizobial symbiosis. The need to address multiple stressors in combination, a concept termed multifactorial stress combinations' (MFSC) whereby three or more environmental parameters or stressors affect plants and crops simultaneously or sequentially, is essential if food security is to be met whilst mitigating environmental damage (Jing et al., 2024; Pascual et al., 2022; Peláez-Vico et al., 2024; Webber et al., 2022; Zandalinas and Mittler, 2022). Future work should build on the processes analysed throughout this thesis through the continued inclusion and analysis of multiple variables, to better understand the complex interactions of environmental, management and varietal differences in response to improve the management of P resources and mitigate soybean production against other stressors.

What is the regulatory mechanism driving increased nodule formation following P fertiliser addition, and can this be manipulated to achieve this increase using more sustainable methods? The regulation of phytohormones has been highlighted as one potential regulatory mechanism to control nodule formation, with soil P deficiency (or other stressors) upregulating the production or inhibition of these hormones (Ferguson et al., 2014; Nagatoshi et al., 2023; Sun et al., 2016). Phosphorus deficiency can increase ethylene production or alter ethylene sensitivity, with increased ethylene production having a predominately negative effect on infection thread formation and nodule development (Borch et al., 1999; Iqbal et al., 2013; Nukui et al., 2000). Abscisic acid (ABA) and cytokinin act antagonistically to regulate nodule initiation, with exogenous ABA inhibiting rhizobial infection and nodulation and

exogenous cytokinin production inducing nodule primordia and nodule-like structure formation, infection thread elongation, and nodule organogenesis (Heckmann et al., 2011; Liu and Murray, 2016). Phosphorus deficiency is suggested to diminish cytokinin levels, with previous studies showing conflicting evidence of foliar ABA concentrations in response to P limitations (Castro-Valdecantos et al., 2023; Cerutti and Delatorre, 2013). Untangling the mechanistic response driving the increased nodule formation following P fertiliser addition opens the potential for this mechanistic response to be manipulated by the alteration of the soil microbiome using more sustainable methods such as the use of beneficial microorganisms or exogenous hormone application.

Alongside the three questions posed above, future work needs to ensure the dissemination of efficient and scientifically sound P fertiliser recommendations to farmers, growers and breeders globally to ensure the proposed increases in soybean yield by P fertiliser addition can be achieved without detriment associated with excess P fertiliser use. Further research should focus on building on the conceptual models of soybean response proposed in Figure 6.1. to develop crop models specific to soybean that capture the complexity of response to P fertiliser addition. The inclusion of processes such as N_2 fixation combined with nutrient stocks and flows throughout the growing season will allow for the improved prediction of soybean response in crop models. More comprehensive analysis (including seed quality, nutrient stocks and flows both within the plant and soil) of soybean response under contrasting P fertiliser treatments in field trials will allow for model development and testing across a wide range of environmental and management conditions. To ensure the sustainable use of P fertiliser within soybean production to successfully balance the requirements to close yield gaps and meet global demand whilst mitigating environmental and economic costs requires the development of clear and economically viable management strategies and guidelines. Interdisciplinary collaboration needs to continue to bridge the commonly occurring knowledge gap between science, policy and practice through engagement and collaboration with farmers to ensure the adaptation of sustainable P fertiliser use to increase soybean production.

7. References

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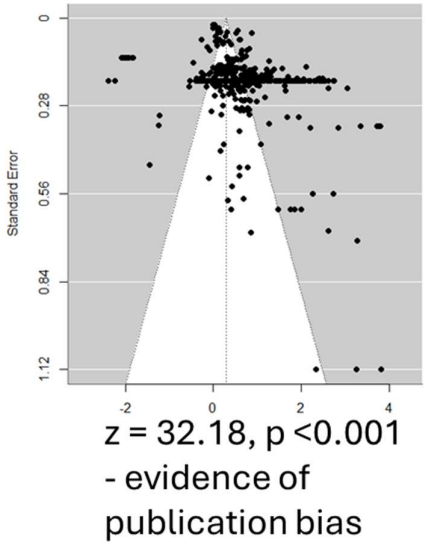
8. Appendix A

Appendix A contains corresponding supplementary Information for chapter two - influence of environmental and management factors on the effectiveness of phosphorus fertiliser in improving soybean yield: a global meta-analysis, currently under review (Field Crops Research).



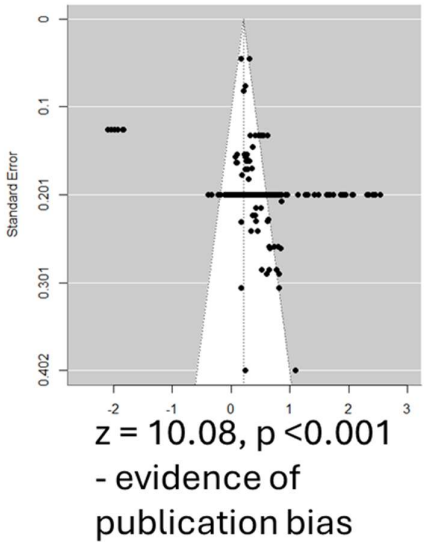


Figure 8.1. Funnel plots and eggers regression showing publication bias for **a.** full dataset, including field, greenhouse and hydroponic studies across all plant response types, and **b.** seed yield *lr* under only field and greenhouse conditions.

Table 8.1. Publications included in data analysis, number of cases (*k*) indicates the number of control/ treatment ratios calculated from each publication.

Publication no.	Author and year.	Publication title	Journal title	No. of cases (*k*)
1	Abdel-Fattah et al., 2014	Influence of arbuscular mycorrhiza and phosphorus fertilization on the gas exchange, growth and phosphatase activity of soybean (*Glycine max* L.) plants	Photosynthetica	6
2	Ulzen et al., 2018	On-farm evaluation and determination of sources of variability of soybean response to *Bradyrhizobium* inoculation and phosphorus fertilizer in northern Ghana	Agriculture, Ecosystems and Environment	4
3	Wang et al., 2020	*mPAP12* is required for nodule development and nitrogen fixation under phosphorus starvation in soybean	Frontiers in Plant Science	3
4	Abbasi et al., 2008	Efficiency of *rhizobium* inoculation and p fertilization in enhancing nodulation, seed yield, and phosphorus use efficiency by field grown soybean under hilly region of rawalakot azad jammu and kashmir, Pakistan	Journal of Plant Nutrition	56
5	Abbasi et al., 2012	Soybean yield and chemical composition in response to phosphorus-potassium nutrition in Kashmir	Agronomy Journal	42
6	Adeyemi et al., 2021	Mycorrhizal growth and phosphorus responses of tropical soybean (*Glycine max* L.) cultivars differ with arbuscular mycorrhizal fungi isolates and phosphorus application rates in a derived-savanna zone of Nigeria	Journal of Plant Nutrition	16

7	Adjei-Nsiah et al., 2021	Influence of phosphorus fertiliser blends on grain yield, nutrient concentration, and profitability of soyabeans in the southern Guinea Savannah of Ghana	South African Journal of Plant and Soil	12
8	Adjei-Nsiah et al., 2019	Influence of p sources and rhizobium inoculation on growth and yield of soybean genotypes on Ferric Lixisols of Northern Guinea Savanna Zone of Ghana	Communications in Soil Science and Plant Analysis	12
9	Adjei-Nsiah et al., 2022	Soybean (*Glycine max* L. Merrill) responds to phosphorus application and *rhizobium* inoculation on Acrisols of the semi-deciduous forest agro-ecological zone of Ghana	Plant Biology	14
10	Thioub et al., 2019	Arbuscular mycorrhizal fungi inoculation enhances phosphorus use efficiency and soybean productivity on a Haplic Acrisol	Soil and Tillage Research	4
11	Ali, 2018	Efficiency of Elemental Sulfur and Phosphorus Fertilizer in Enhancing Soybean (*Glycine max* L.) Growth and Yield in a Clayey Soil	Egyptian Journal of Soil Science	36
12	Antonangelo et al., 2019	Soybean Yield Response to Phosphorus Fertilization in an Oxisol under Long-Term No-Till Management	Soil Fertility and Plant Nutrition	6
13	Awuni et al., 2023	Lime, inoculum, and phosphorous input supplementation under rain-fed soybean in Ghana's northern savannas	Frontiers in Sustainable Food Systems	9
14	Basal and Szabo 2020	Sole and combined effects of drought and phosphorus application on soybean	Journal of Central European Agriculture	8

15	Borges and Mallarino, 2003	Broadcast and deep-band placement of phosphorus and potassium for soybean managed with ridge tillage	Soil Science Society of America Journal	56
16	Bulgarelli et al., 2017	Mycorrhizae enhance nitrogen fixation and photosynthesis in phosphorus starved soybean (Glycine max L. Merrill)	Environmental and Experimental Botany	8
17	Cahyono and Minardi, 2022	Effect of Fast Dissolved Phosphorus Fertilizer on the Growth, Seed Product, and Phosphorus Uptake Efficiency of Soybean (Glycine max L.)	AGRIVITA Journal of Agricultural Science	2
18	Caires et al., 2023	Crop nutrition and grain yield as affected by phosphorus fertilization and continued use of phosphogypsum in an Oxisol under no-till management	Archieves of Agronomy and Soil Science	9
19	Caires et al., 2017	Phosphate fertilization strategies for soybean production after conversion of a degraded pastureland to a no-till cropping system	Geoderma	11
20	Li et al., 2022	Effect of phosphate rock sources on biological nitrogen-fixation by soybean	Agronomy	48
21	Codling, 2019	Effects of phosphorus amended low phosphorus soil on soybean (Glycine max L.) and wheat (Titicum aestivum L.) yield and phosphorus uptake	Journal of Plant Nutrition	6
22	Dabesa and Tana, 2021	Response of Soybean (Glycine max L. (Merrill)) to Bradyrhizobium Inoculation, Lime, and Phosphorus Applications at Bako, Western Ethiopia	International Journal of Agronomy	6

23	Eberhardt et al., 2017	Phosphorus bioavailability in soybean grown after pasture under different fertility regimes	*Semina: Ciências Agrárias*	4
24	Egamberdieva et al., 2018	Interactive Effects of Nutrients and Bradyrhizobium japonicum on the Growth and Root Architecture of Soybean (Glycine max L.)	Frontiers in Microbiology	4
25	Fageria et al., 2014	Phosphorus Nutrition of Upland Rice, Dry Bean, Soybean, and Corn Grown on an Oxisol	Communications in Soil Science and Plant Analysis	8
26	Fageria et al., 2011	Response of Soybean to Phosphorus Fertilization in Brazilian Oxisol	Communications in Soil Science and Plant Analysis	27
27	Fang et al., 2011	Crop Root Behavior Coordinates Phosphorus Status and Neighbors: From Field Studies to Three-Dimensional in Situ Reconstruction of Root System Architecture	Plant Physiology	2
28	Faozi et al., 2019	Effectiveness of phosphorus fertilizer on soybean plants in the coastal sands soil	IOP Conference Series: Earth and Environmental Science	36
29	Fernández et al., 2009	Compared Phosphorus Efficiency in Soybean, Sunflower and Maize	Journal of Plant Nutrition and Soil Science	10
30	Fernández et al., 2011	Effect of indigenous mycorrhizal colonization on phosphorus-acquisition efficiency in soybean and sunflower	Journal of Plant Nutrition and Soil Science	4
31	Fernández and Rubio, 2015	Root morphological traits related to phosphorus-uptake efficiency of soybean, sunflower, and maize	Journal of Plant Nutrition and Soil Science	6

32	Gonyane and Sebetha, 2022	The Effect of Plant Density, Zinc Added to Phosphorus Fertilizer Sources and Location on Selected Yield Parameters of Soybean	Legume Research	4
33	Hankinson et al., 2015	Effect of Planting Date and Starter Fertilizer on Soybean Grain Yield	Crop, Forgage & Turfgasss Management	6
34	He et al., 2019	Phosphorus application increases root growth, improves daily water use during the reproductive stage, and increases grain yield in soybean subjected to water shortage	Environmental and Experimental Botany	16
35	He et al., 2013	Profiling of microbial PLFAs: Implications for interspecific interactions due to intercropping which increase phosphorus uptake in phosphorus limited acidic soils	Soil Biology and Biochemistry	2
36	Janegitz et al., 2016	Brachiaria as a Cover Crop to Improve Phosphorus Use Efficiency in a No-till Oxisol	Revista Brasileira de Ciência do Solo	8
37	Jemo et al., 2008	Biological nitrogen fixation potential by soybeans in two low-P soils of southern Cameroon	Nutrient Cycling in Agroecosystems	12
38	Jin et al., 2022	Nodule Formation and Nitrogen Use Efficiency Are Important for Soybean to Adapt to Water and P Deficit Conditions	Agronomy	8
39	Kakiuchi and Kamiji, 2014	Relationship between Phosphorus Accumulation and Dry Matter Production in Soybeans	Agronomy & Crop Ecology	110
40	Kamran et al., 2018	Effect of different phosphorus sources on soybean growth and arsenic uptake under arsenic stress conditions in an acidic ultisol	Ecotoxicology and Environmental Safety	12

41	Karaca et al., 2013	Effects of Mycorrhizae and Fertilization on Soybean Yield and Nutrient Uptake	Communications in Soil Science and Plant Analysis	16
42	Kolawole, 2012	Effect of phosphorus fertilizer application on the performance of maize/soybean intercrop in the southern Guinea savanna of Nigeria	Archieves of Agronomy and Soil Science	8
43	Li et al., 2022	Effect of Phosphorus Supply Levels on Nodule Nitrogen Fixation and Nitrogen Accumulation in Soybean (Glycine max L.)	Agronomy	42
44	Lyu et al., 2016	Major Crop Species Show Differential Balance between Root Morphological and Physiological Responses to Variable Phosphorus Supply	Frontiers in Plant Science	2
45	Mahamood et al., 2009	Comparative growth and grain yield responses of soybean genotypes to phosphorous fertilizer application	African Journal of Biotechnology	24
46	Mahanta et al., 2018	Modification of root properties with phosphate solubilizing bacteria and arbuscular mycorrhiza to reduce rock phosphate application in soybean-wheat cropping system	Ecological Engineering	12
47	Mallarino et al., 2009	Timing of Broadcast Phosphorus Fertilization for No-Till Corn and Soybean	Soil Science Society of America Journal	100
48	Medina et al., 2022	Limitations in Grain Yield and Carbon Partitioning Differs in Soybean (Glycine max (L.) Merr.) Cultivars with Contrasting Photosynthetic Phosphorus-Use Efficiency	Journal of Soil Science and Plant Nutrition	6
49	Messiga et al., 2012	Long term impact of tillage practices and biennial P and N fertilization on maize and soybean yields and soil P status	Field Crops Research	12

50	Mirriam et al.,2022	Aggrandizing soybean yield, phosphorus use efficiency and economic returns under phosphatic fertilizer application and inoculation with Bradyrhizobium	Journal of Soil Science and Plant Nutrition	36
51	Moreira et al., 2020	Phosphate Fertilizer in Soybean-Wheat Cropping System Under No-Till Management	Frontiers in Sustainable Food Systems	12
52	Moreira et al., 2013	Agronomic Efficiency of Two Types of Lime and Phosphate Fertilizer Sources in Brazilian Cerrado Soils Cultivated with Soybean	Communications in Soil Science and Plant Analysis	18
53	Ogoke et al., 2003	Maturity Class and P Effects on Soya Bean Grain Yield in the Moist Savanna of West Africa	Journal of Agronomy and Crop Science	4
54	Olibone and Rosolem, 2010	Phosphate fertilization and phosphorus forms in an Oxisol under no-till	Soils and Plant Nutrition	2
55	Qin et al., 2022	Increased nodular P level induced by intercropping stimulated nodulation in soybean under phosphorus deficiency	Nature Scientific Reports	8
56	Rotaru and Sinclair, 2009	Interactive influence of phosphorus and iron on nitrogen fixation by soybean	Environmental and Experimental Botany	10
57	Rurangwa et al., 2018	Benefits of inoculation, P fertilizer and manure on yields of common bean and soybean also increase yield of subsequent maize	Agriculture, Ecosystems and Environment	6
58	Sajwan et al., 2006	Phosphorus alleviation of cadmium phytotoxicity	Journal of Plant Nutrition	18
59	Salim et al., 2023	Phosphorus Application Enhances Root Traits, Root Exudation, Phosphorus Use Efficiency, and Seed Yield of Soybean Genotypes	Plants	38

60	Savin et al., 2009	Response of Mycorrhizal Infection to Glyphosate Applications and P Fertilization in Glyphosate-Tolerant Soybean, Maize, and Cotton	Journal of Plant Nutrition	3
61	Sha et al., 2019	A Reduced Phosphorus Application Rate Using a Mycorrhizal Plant as the Preceding Crop Maintains Soybean Seeds' Nutritional Quality	Journal of Agricultural and Food Chemistry	4
62	Singh et al., 2019	Phosphorus and Potassium Fertilizer Rate Verification for a Corn-Wheat-Soybean Rotation System in Tennessee	Agronomy Journal	16
63	Spagnoletti et al., 2018	Phosphorus fertilization reduces the severity of charcoal rot (Macrophomina phaseolina) and the arbuscular mycorrhizal protection in soybean	Journal of Plant Nutrition and Soil Science	12
64	Thioub et al., 2019	Arbuscular mycorrhizal fungi inoculation enhances phosphorus use efficiency and soybean productivity on a Haplic Acrisol	Soil and Tillage Research	8
65	Vieira et al., 2022	Soybean Yield, Yield Components, and Phosphorus Concentration Under Different Phosphate Sources	Communications in Soil Science and Plant Analysis	48
66	Wasike et al., 2009	Genetic diversity of indigenous Bradyrhizobium nodulating promiscuous soybean Glycine max (L) Merr. varieties in Kenya: Impact of phosphorus and lime fertilization in two contrasting sites	Plant and Soil	32
67	Widodo et al., 2022	Effect of phosphate-solubilizing bacteria isolates in acid soil on soybean (Glycine max L.) seed yield	Applied Ecology and Environmental Researc	16
68	Xing et al., 2022	GmSPX8, a nodule-localized regulator confers nodule development and nitrogen fixation under phosphorus starvation in soybean	BMC Plant Biology	2

69	Yang et al., 2022	Both biomass accumulation and harvest index drive the yield improvements in soybean at high and low phosphorus in south-west China	Field Crops Research	6
70	Yao et al., 2018	Variation of nitrogen accumulation and yield in response to phosphorus nutrition of soybean (Glycine max L. Merr.)	Journal of Plant Nutrition	18
71	Yu et al., 2020	Responses in Zinc Uptake of Different Mycorrhizal and Non-mycorrhizal Crops to Varied Levels of Phosphorus and Zinc Applications	Frontiers in Plant Science	16
72	Zhao et al., 2022	Phosphate fertilizers increase CO2 assimilation and yield of soybean in a shaded environment	Photosynthetica	2
73	Zhu et al., 2020	Physiological Response of Phosphorus-Efficient and Inefficient Soybean Genotypes under Phosphorus-Deficiency	Russian Journal of Plant Physiology	8
74	Zhu et al., 2021	A phosphate starvation responsive malate dehydrogenase, GmMDH12 mediates malate synthesis and nodule size in soybean (Glycine max)	Environmental and Experimental Botany	2

Table 8.2. ANOVA table of explanatory variables included in main model, and the exclusion of test explanatory variable. Explanatory variable categories have been included where relevant. The main model had a significant QE ($p < 0.001$), meaning there is evidence of underlying heterogeneity in the data that cannot be explained by explanatory variables. All included explanatory variables were significant (QM $p < 0.001$), and all (except inoculant and soil available phosphorus) were significant when compared to the full model and model without corresponding test moderator (LRT *p* value).

Explanatory variable removed	**QE**			**QM**			**LRT**		
k = 459		*p* value	df		*p* value	df		*p* value	df
Main model	1528.19	<0.001	396	2320.32	<0.001	62			
Year	1786.16	<0.001	419	2044.83	<0.001	39	258.97	<0.001	40
Country	1613.43	<0.001	407	2217.56	<0.001	51	85.24	<0.001	52
Experimental system	1551.74	<0.001	397	2279.25	<0.001	61	23.56	<0.001	62
Inoculant	1530.12	<0.001	339	2300.87	<0.001	59	1.94	0.586	60
Fertiliser rate	1551.25	<0.001	398	2279.74	<0.001	60	23.06	<0.001	60

(< 40 kg P/ha, > 40 kg P/ ha)									

9. Appendix B

Appendix B contains supporting information relevant to Chapter Three, including rational, materials and methods and extended results and discussion.

9.1. Rational

The process of nitrogen (N_2) fixation reduces reliance on synthetic N fertilisers and the likelihood of crop N deficiency, improving yields and reducing environmental and economic costs (Gao et al., 2023). Reliable estimation of N_2 fixation is essential to guide management decisions to improve soybean N_2 fixation and yield, with N limitation being the primarily limiting resource for soybean yield after water deficit water (Giller and Cadisch, 1995; Hirel et al., 2007; Sinclair and Wit, 1975). Multiple methods have been proposed to allow for the estimation of N_2 fixation, including direct estimations such as $\delta^{15}N$ isotope dilution or $\delta^{15}N$ natural abundance technique ($\delta^{15}N$ NA), abundance of relative ureide (RAU) techniques of both stem tissue and xylem sap or acetylene reduction assays; each with their own advantages and disadvantages (Unkovich and Pate, 2000). Isotope based methods give a time-integrated measure of the total amount of N fixed over the entire growth period; however, these methods rely on the growth of an appropriate reference plant or isotope dosing, involving labour intensive and expensive sample preparation and determination (Schweiger et al., 2012; Thilakarathna et al., 2018). The RAU method requires less expensive and sophisticated equipment, and hence in theory can be utilised as a reliable predictor of N fixation processes, with the accuracy confirmed using benchmark techniques (Herridge et al., 1990; Rosso et al., 2021). However, RAU analysis gives only a measure of N_2 derived from fixation (Ndfa) at the sampling point (Herridge and Peoples, 2002). Relative abundance of ureide analysis can be transformed to Ndfa using regression equations developed using calibration with $\delta^{15}N$ NA techniques as developed by Herridge et al. (1990). There are a number of variables that can cause potential sources of error with this method; and whilst the effect of P stress is unknown on these relationships, other abiotic stressors, such as drought, N stress and plant senescence are known to affect the relationship (Aveline et al., 1995; Purcell et al., 2004). In stem extracted RAU, some differences in relationships between RAU and Ndfa of varieties was observed, with a trend for the early-maturing genotypes to have higher RAU values at the same level of Ndfa (Herridge and Peoples, 1990).

Alongside accurate estimation of N_2 fixation in soybean, there is a need to improve understanding of nutrient behaviour to improve fertiliser recommendations and optimise soybean yield and N_2 fixation (Edreira et al., 2017; Rotaru and Sinclair, 2009). Whilst patterns of nutrient uptake, partitioning and remobilisation were studied in the 1930s – 70s to better understand the physiology of nutrient accumulation, rapidly changing management practices and genetic advancement in breeding may favour differences in nutrient uptake and remobilisation than originally thought (Bender et al., 2015). Phosphorus fertiliser addition is required for the process of N_2 fixation to optimise nodule formation and maintaining functional nodule tissue and the provision of ATP (Li et al., 2018). Whilst critical soil P test levels for soybean production have been estimated to be approximately 9 mg P kg^{-1} Olsen's extractable P, this is dependent on environmental and management factors, and the critical

levels for nodulation and N_2 fixation response are less well understood (Culman et al., 2020; Scott et al., 1987).

This study aimed to evaluate the RAU method for quantifying N_2 fixation and soybean N_2 fixation and growth response under differing environmental conditions and experimental designs to ensure successful soybean growth and N2 fixation response to P fertiliser addition could be observed and quantified in future studies throughout the thesis.

9.2. Materials and Methods

9.2.1. Field trial experimental design and harvest

A field trial was conducted in collaboration with the experimental station of the Leibniz Centre for Agricultural Landscape Research (ZALF) in Müncheberg (52°31'N, 14°07'E, 62 m above sea level), 50 km east of Berlin, in 2022. Soils at the research station are predominantly sandy loams and loamy sands with high spatial heterogeneity, however, in this case there were no significant differences between soil characteristics (Table 9.1.). The long-term average annual temperature is 9.0 °C and average annual precipitation is 563 mm (Karges et al., 2022). Four soybean genotypes of three different maturity groups – RGT Siroca (00 – late), Merlin (000 – early), Tofina (000 – early), SY Livius (0000 – very early) and a buckwheat control crop were grown in randomised split plot design with four replicates. All plants were sown to a planting density of 70 – 80 visible grains m^{-2} and a depth of 3 - 4 cm in 8 x 3 m plots comprising of 6 rows spaced 0.5 m apart. Prior to sowing, seeds were inoculated with Histick Soy (BASF Services Europe, Berlin, Germany), an on-farm peat-based seed inoculant at a density of 2 x 10^9 colony forming units (CFU) *B. japonicum* at an application rate 400 g 100 kg^{-1} seed.

Table 9.1. Field trial soil characteristics (0 – 15 cm) under different experimental plots calculated from five pseudo-replicates across each of the four experimental plots for each variety, Merlin, RGT Siroca, SY Livius and Tofina. Data is reported as mean ± standard error. One-way ANOVA *p* values are displayed.

	Merlin	**RGT Siroca**	**SY Livius**	**Tofina**	***p* value**
Plant available phosphorus (mg kg^{-1})	7.96 ± 0.87	8.59 ± 3.04	12.66 ± 4.36	6.79 ± 1.35	0.496 *ns*
Plant available potassium (mg kg^{-1})	13.59 ± 0.87	12.47 ± 3.04	10.37 ± 4.36	12.46 ± 1.35	0.423 *ns*
Plant available ammonium (mg kg^{-1})	0.14 ± 0.02	0.13 ± 0.01	0.19 ± 0.02	0.14 ± 0.01	0.097 *ns*
Plant available nitrate (mg kg^{-1})	0.73 ± 0.12	0.82 ± 0.12	1.17 ± 0.24	0.73 ± 0.26	0.379 *ns*
Total N (%)	0.09 ± 0.00	0.09 ± 0.00	0.1 ± 0.01	0.09 ± 0.01	0.746 *ns*
Total C (%)	1.05 ± 0.02	1.09 ± 0.04	1.13 ± 0.04	1.14 ± 0.07	0.554 *ns*
pH	6.14 ± 0.35	6.04 ± 0.23	6.17 ± 0.31	5.91 ± 0.22	0.915 *ns*

Plant samples were collected by hand, when soybean reached full flowering (R2) based on physiological development, from a randomly selected 0.5 m^2 area from the internal rows of the plot up to 1 m depth, with the outer two rows removed for edging effects. Following harvest, plant samples

were bulked by plot and partitioned into stem, leaf, root and nodules. Nodules were counted by hand, and partitioned plant tissues were weighed to obtain fresh weight and dried at 40°C for at least 72 h to obtain dry biomass weight.

9.2.2. Controlled environment study - experimental design and harvest

One soybean genotype (Merlin) was grown under two P treatments with 14 replicates of each treatment, and 14 replicates of maize were grown under controlled P treatments as reference plants, all in 0.75 l pots. These pots were made from cylindrical plastic tubing (90 mm diameter x 200 mm height) with a steel mesh base designed to fit into a Scholander type pressure chamber (SoilMoisture Equipment Corp., Santa Barbara, CA, USA) for the collection of root-bleeding xylem sap. The pots were filled with a 3:1 mixture of sandy loam topsoil and Leighton buzzard lime free sand (Table 9.2.), with granular magnesium and potassium fertilisers applied to all pots at rates of 0.025 and 0.06 g pot^{-1} respectively (18 and 86 kg ha^{-1} equivalent). Phosphorus fertiliser (single superphosphate, 18% P_2O_5) was applied at a rate of 0.11 g P pot^{-1}, equivalent to 60 kg P ha^{-1} to the P addition treatments, with no P fertiliser being applied to the control treatment.

Table 9.2. Soil characteristics prior to any fertiliser additions calculated from a subsample comprising 10 pseudo-replicates of the mixed sand: soil prior to fertiliser treatment and pot filling. Soil analysis methods are given in section 9.2.6. below.

Pre-treatment soil characteristics	
Sand content (%)	91
Plant available phosphorus (mg kg^{-1})	6
Plant available ammonium (mg kg^{-1})	1
Plant available nitrate (mg kg^{-1})	21
pH	7

Prior to sowing, seeds were first sterilised by immersion in 70% ethanol for 45 s, rinsed in sterile water, immersed in 5% NaHCl for five minutes, then rinsed in sterile water five times. Following sterilisation, seeds were pre-germinated on sterile filter paper. Following successful germination, seeds were selected for uniformity with one seed being transplanted at 3 cm depth in the centre of 0.75 l pots. On transplanting, seeds under inoculated treatments were inoculated with 1 ml *B. japonicum* USDA110 at a density of 1 x 10^8 CFU. USDA110 was previously cultured on MAG agar for 7 days at 30°C. Following successful emergence, at ten days, healthy plants were thinned to one per pot for uniformity.

Plants were watered daily to maintain a water holding capacity between 70 & 80%. Plants were grown in a CE room at Lancaster University, with an average day/night temperature 25/18°C and a 16-hour photoperiod (8:00 - 24:00, with a 30-minute ramping period between 8:00 and 8:30). Micronutrients were applied in the form of an adapted N and P free Hoagland's solution (Table 9.3) at a rate of 1 ml l^{-1} of water applied at day ten and biweekly until harvest.

Table 9.3. Adapted (nitrogen and phosphorus free) Hoaglands solution

Reagent	Concentration (mg l^{-1})
Boric Acid	2.86
Manganese Sulphate	1.36
Zinc Sulphate	0.22
Copper Sulphate	0.008
Sodium Molybdate	0.025
Iron (III) Chloride	0.16

Plants were harvested when soybean reached the beginning of flowering (R1) according to average physiological development. Seven replicates of each treatment were randomly selected to be used for root xylem sap collection. Plants were de-topped below the cotyledons, leaving 8 cm of stem protruding above the soil. The whole pot was sealed in a Scholander pressure chamber, and the cut surface of the shoot was rinsed with di water and dried with clean tissue to remove contamination from damaged cells. Sap samples were weighed until generated flow rates matched average pre-determined *in planta* sap flow rates. Whole plant transpiration rate was determined gravimetrically prior to harvest by sealing the top and base of the pot and dividing weight loss by time to determine average *in planta* sap flow rate. Once generated flow rates matched average pre-determined average *in planta* sap flow rate, a 0.1 ml sample was taken and immediately frozen (-20 °C) until RAU analysis was completed. Care was taken to ensure xylem sap was collected within 30 minutes of de-topping as outside of this timeframe ureide concentrations destabilise (Herridge et al., 1988). The remaining seven replicates were not used to collect root xylem sap, rather, plants were de-topped and immediately partitioned for biomass analysis and stem samples collected for RAU analysis.

At harvest, plants were de-topped and below-ground material was washed to remove any soil, then nodules were removed and counted by hand. This process was completed sequentially for all plants harvested. All partitioned plant tissues were weighed to obtain fresh weight and dried at 40°C for at least 72 h to obtain dry biomass weight.

9.2.3. Nitrogen fixation analysis

Relative Ureide Assay

Dried stem samples of soybean were milled to <1 mm diameter using a ball mill for relative abundance of ureide (RAU) analysis. 0.1 g of stem tissue was extracted in 5 ml 0.1 mol l^{-1} phosphate buffer and 2.5 ml ethanol. Extracts were then vortexed and heated to 80°C in a water bath for five minutes. Extracts were cooled to room temperature (25 minutes) then centrifuged at 3000 rpm for five minutes and the supernatant filtered through Whatman no. 2 filter paper.

Adapted Young-Conway's (Young and Conway, 1942) method was used to determine ureide N using colorimetry and spectrophotometer (Ultrospec 2100 pro, Biochrom Ltd., Cambridge, UK). Extracted stem samples went through a 25 times dilution using DI water. To this, 0.5 ml 0.5M sodium hydroxide was added before extracts were vortexed and heated to 100°C in a water bath for 15 minutes. Extracts were cooled to room temperature (25 minutes) then 0.5ml 0.65M HCl and 0.5ml phenylhydrazine hydrochloride was added before extracts were vortexed and heated to 100°C in a water bath for two minutes 40 seconds. Extracts were immediately removed and placed in an ice bath for 15 minutes before 2 ml concentrated hydrochloric acid and 0.5 ml potassium ferricyanide was added and extracts vortexed immediately. Extracts were then read at 525 nm within 10 – 30 minutes of the assay. Calibration standards consisting of allantoin, ethanol and di water were analysed at 0, 0.025, 0.05, 0.075 and 0.125 µMol.

Adapted ninhydrin (Yemm and Cocking, 1955) methods were used to determine nitrate N using colorimetry and spectrophotometer. 100 µl of extract was added to 0.4 ml of salicylic acid, vortexed and left at room temperature for 20 minutes. 9.5 ml 2M sodium hydroxide was added, vortexed and left at room temperature for 15 minutes before absorbance was read at 410 nm within 30 minutes.

From this relative ureide was calculated:

$$Relative\ Abundance\ of\ Ureide_N\%(RAU\%) = \left(\frac{4U}{4U+N}\right) \times 100$$

Where U and N are concentrations of ureide and nitrate (Herridge and Peoples, 1990). Nitrogen derived from fixation (Ndfa%) was calculated based on quadratic function proposed by Herridge and Peoples (1990):

$$RAU\% = 1.35 + 0.311Ndfa\% + 0.0057\ Ndfa\%^2$$

Xylem sap collected from the subsampled treatments in controlled environment study two were used to determine ureide and nitrate N using colorimetry and spectrophotometer following the adapted Young-Conway's (Young and Conway, 1942) and ninhydrin (Yemm and Cocking, 1955) methods described above. Adpated Cataldo (Cataldo et al., 1975) methods were used to determine amino acid N using colorimetry and spectrophotometer. Xylem sap samples went through a 10 times dilution using DI water. To this, 0.5 ml of citrate buffer was added and extracts vortexed. 1.3 ml of ninhydrin reagent was then added, extracts were then vortexed and heated to 100°C in a water bath for 15 minutes. Extracts were cooled to room temperature (15 minutes) then 3 ml ethanol was added and absorbance read at 570 nm within 30 minutes.

From this relative ureide was calculated as Herridge and Peoples (1990):

$$RAU\% = \left(\frac{4U}{4U+N+AA}\right) \times 100$$

Where U, N and AA are concentrations of ureide, nitrate and Amino acids (Herridge and Peoples, 1990). Nitrogen derived from fixation was calculated based on linear function proposed by Herridge and Peoples (1990):

$$RAU\% = 4.8 + 0.83Ndfa\%$$

$\delta^{15}N$ natural abundance

For $\delta^{15}N$ NA methods the use of a reference crop, grown in the same experimental conditions is required. In the field trial, buckwheat was grown in experimental plots identical to the soybean plots, with four randomly distributed blocks within the field, and for the second controlled environment study maize was grown under controlled P treatments in 0.75 L pots. Oven dried and milled stem and leaf samples were bulked according to dry weight ratios to allow for the shoot tissue analysis. Shoot tissue was analysed for carbon (C) and N concentration and isotopic composition; subsamples were weighed into tin capsules for isotopic ratio mass spectrometry. Nitrogen fixation rates were calculated using the standard equation based on N isotopic data of plant shoots (Unkovich et al., 1994):

$$\%Ndfa = \left(\frac{\delta 15N_{reference} - \delta 15N_{soybean}}{\delta 15N_{reference} - B}\right) \times 100$$

Where $^{15}N_{reference}$ and $^{15}N_{soybean}$ are the natural abundance of the reference (buckwheat and maize for field trial and controlled environment study data respectively) and soybean plants. '*B*' is the shoot $\delta^{15}N$ of soybeans fully reliant on N_2 fixation for uptake, a value of -1.76 was used in line with the mean *B* value compiled through a recently published literature review (Balboa et al., 2019).

9.2.4. Statistical analysis

Outliers were determined if greater than two standard deviations from the mean and removed from the dataset. Data were tested for normality using Shapiro-Wilk test and a visual assessment of histograms and Q-Q plots. Where data was not normally distributed, data was transformed, using either logarithmic or square root functions to achieve normality. A one-way analysis of variance (ANOVA) assessed for statistical differences in plant response and nutrient stocks in the field trial. Two-way ANOVA assessed for statistical differences in plant response, with P treatment and light intensity as factors in the first CE study and P treatment and inoculation as factors in the second CE study. To assess pairwise differences, a Tukey's HSD post hoc test was applied.

Relationships between Ndfa determined from $\delta^{15}N$ NA and RAU methods, in both the field and controlled environment study datasets, were assessed using linear regression analysis. The predetermined quadratic function from Herridge and Peoples (1990) for stem RAU analysis, and the linear function for xylem sap analysis was used to model the relationship between RAU and Ndfa determined from $\delta^{15}N$ NA for the field and CE study data. The fit of this function was evaluated using the coefficient of determination (R^2) and mean squared error (mse).

9.2.5. Field trial soil analysis

Five pseudo-replicates of soil samples were taken at the R2 phase of phenological development to a depth 0 - 15 cm within each experimental plot, all soil analysis was completed by collaborators at the central laboratories at ZALF. Plant available P and K were extracted using double lactate method, and plant available ammonium and nitrate, and pH extracted using potassium chloride, total N and C was measured using an Elemental Vario EL CUBE CN analyser. Mean and standard error across the four varieties of soybean was calculated along with a one-way ANOVA to ensure no statistical differences between soil characteristics.

9.2.6. Controlled environment study pre-treatment soil analysis

Particle size distribution analysis was based on methods established by Gale & Hoare (1991). Two grams of air-dried soil was digested in approximately 20 ml hydrogen peroxide (H_2O_2) and heated to 75°C. Additional H_2O_2 was added until no further reaction occurred, 1 ml of 10% Calgon (10% weight: volume sodium exametaphosphate ($(NaPO_3)_6$)) was added, the mixture stirred and then left overnight. Sample analysis was performed in triplicate using a LS13320 Particle Size Analyzer (Beckman Coulter) with the laser set to 780 nm and the PIDS set to 462, 604 and 900. To measure pH, 10 g of air-dried soil was mixed with 25 ml of de-ionised water in a 50 ml centrifuge tube, stirred and left to stand for an hour. The sample was then tested using an Orion™ Model 91-72 Sure-Flow pH Electrode (Thermo Fisher Scientific Inc., Waltham, USA) and pH meter (Denver Instruments, Bohemia, New York, USA).

Extraction for plant available ammonium and nitrate was undertaken using 2 M KCl as an extraction matrix. Five grams of fresh soil was mixed with 25 mL KCl (1 M), shaken on an orbital shaker for 1 hour at 180 rpm. Samples were then centrifuged at 3000 rpm for five minutes and the supernatant filtered through Whatman no. 2 filter paper. The filtrate was analysed for nitrate and ammonium using a colorimetric segmented flow analyser (AA3, Seal Analytical, Southampton, UK).

Extraction for plant available P was undertaken using Olsen reagent (0.5 M $NaHCO_3$ adjusted to pH 8.5 with 10 M NaOH solution) as an extraction matrix. One gram of air-dried and 2 mm sieved soil was mixed with 20 ml of Olsen reagent, shaken on a mechanical end over end shaker for 30 minutes. Samples were then centrifuged at 3000 rpm for five minutes and the supernatant filtered through Whatman no. 2 filter paper. All extracts went through a ten times dilution with deionised (DI) water before extractable P was determined using a microplate reader (BMG Labtech, Ortenberg, Germany). A mixed reagent was made up on the day of analysis comprising of 10 mL reagent A (2.5 M H_2SO_4), 3 mL reagent B (1 g of ammonium molybdate diluted in 25 mL of de-ionised water), 1 mL reagent C (0.07 g of potassium antimonytartrate diluted in 25 mL of de-ionised water) and 6 mL reagent D (0.44 g of ascorbic acid diluted in 25 ml de-ionised water). Reagent D was made on the day of analysis. 200 µl of the sample extract or standard was added to a microplate well, followed by 40 µl of mixed reagent and left to stand at room temperature for 20 minutes. Once the colour had fully

developed, the samples were read at an absorbance of 880 nm within 40 minutes of the mixed reagent being added. Calibration standards for each microplate were analysed at 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1 ppm.

9.3. Results and Discussion

9.3.1. Field trial: nitrogen fixation, nodulation and nutrient stock response of different varieties

Whilst plant dry weight did not show significant (p = 0.087) differences between varieties, Merlin had a trend of increased biomass production at the R2 phase of phenological development (Table 9.4). Nodule number and nodule dry weight both significantly ($p < 0.05$) differed between varieties. RGT Siroca had a significantly ($p < 0.05$) greater plant nodule number than Merlin and Tofina, with SY Livius having a significantly ($p < 0.05$) greater nodule dry weight than Tofina. Despite this, no significant (p = 0.119) differences were observed in RAU. Limited statistical differences were observed in the whole plant and partitioned plant nutrient stocks at the R2 phase of phenological development (Table 9.5); however significant differences (p <0.05) were observed in plant N stocks, with Merlin plant N stocks were significantly ($p < 0.05$) greater than the other varieties and Merlin leaf N stocks significantly ($p < 0.05$) greater than Tofina. Whilst not significant (p = 0.064), leaf P stocks varied across varieties, with Merlin and SY Livius having larger stocks than RGT Siroca and Tofina.

Whilst significant ($p < 0.05$) differences were observed in the nodulation of the four varieties (Table 9.4), the rates of nodulation were objectively very low compared to other studies of soybean cultivation within Europe (e.g. Soboko et al., 2020). However, the lower rate of nodulation was also observed in other experiments in similar locations, when plants were inoculated with other varieties of rhizobia (Omari et al., 2022), and when plants were inoculated with the same inoculant (Nartey et al., 2022). This low rate of nodulation can be reflected in the low percentages of RAU, (15 – 20%), with values being low compared to those generally reported, particularly given the soil is N limited (Herridge et al., 2008b; Salvagiotti et al., 2008; Schipanski et al., 2010). These results suggest the use of the commercial inoculant was not very successful, or another factor was limiting nodulation and N_2 fixation, particularly when compared to the RAU and nodulation recorded in the CE studies (Table XX). This low N_2 fixation may be due to constraining environmental conditions at this latitude, including temperature, photoperiod or irradiance (Vollmann et al., 2000), or other soil nutrients (such as P) limiting nodulation and N_2 fixation processes.

Whilst not significant (p = 0.087), Merlin had the largest plant biomass and nitrogen stocks and RGT Siroca the smallest, suggesting potential differences in growth mechanisms and nutrient partitioning that may respond differently to P fertiliser addition. This result contributed to the decision to take these varieties forward for further experimental work. The significant ($p < 0.05$) differences in nodule number and dry weight between Merlin and SY Livius would have made these two varieties interesting to take forward and subject to differing P fertiliser treatments, however, it was not possible to source SY Livius seed. These varieties do not have significantly different P stocks, suggesting similar PUE and P fertiliser requirements, however further work needs undertaking to determine critical thresholds for

these varieties and examine how they respond to P fertiliser addition in Europe-centred systems. Interestingly, whilst not significant, Merlin had increased P stocks in whole plant, leaf, root and stem, suggesting potentially increased P uptake and or P use efficiency.

Table 9.4. Biomass, nodule number, nodule dry weight and relative abundance of ureide (RAU) of different varieties (Merlin, RGT Siroca, SY Livius, and Tofina) in the field experiment. An area of 0.5 m^2 randomly selected within each plot was harvested at the R2 phase of phenological development. Data are means ± SE of 4 replicates. Results of one-way ANOVA are displayed, with letters of significance indicating Tukeys post-hoc results.

Source of Variation	RAU %	Plant biomass (g m^{-3})	Nodule number (No. plant $^{-1}$)	Nodule dry weight (mg $plant^{-1}$)
Merlin	16.7 ± 0.1	426.9 ± 30.7	11.2 ± 1.6 b	16 ± 1 ab
RGT Siroca	17.0 ± 0.7	339.1 ± 14.1	15.9± 2.2 a	18 ± 2 ab
SY Livius	15.5 ± 1.8	365.9 ± 30.6	12.7± 1.7 ab	21 ± 3 a
Tofina	20.8 ± 1.6	351.2± 10.7	6.8 ± 1.9 b	11 ± 5 b
Variety (V)	0.119	0.087	< 0.05	< 0.05

Table 9.5. Partitioned plant tissue nutrient stocks of different varieties (Merlin, RGT Siroca, SY Livius, and Tofina) in the field experiment. An area of 0.5 m^2 randomly selected within each plot was harvested at the R2 phase of phenological development. Data are means ± SE of 4 replicates. One-way ANOVA *p* values are displayed, with letters of significance indicating Tukeys post-hoc results.

Source of Variation	Plant N (g m^{-2})	Plant P (g m^{-2})	Plant K (g m^{-2})	Leaf N (g m^{-2})	Leaf P (g m^{-2})	Leaf K (g m^{-2})	Stem N (g m^{-2})	Stem P (g m^{-3})	Stem K (g m^{-3})	Root N (g m^{-3})	Root P (g m^{-3})	Root K (g m^{-3})
Merlin	10.41 ± 0.42 a	0.74 ± 0.06	8.60 ± 0.53	6.71 ± 0.37 a	0.40 ± 0.04	3.14 ± 0.16	3.05 ± 0.11	0.27 ± 0.03	4.51 ± 0.41	0.65 ± 0.06	0.07 ± 0.00	0.95 ± 0.04
SY Livius	8.05 ± 0.33 b	0.65 ± 0.05	7.08 ± 0.58	5.35 ± 0.23 ab	0.35 ± 0.03	2.84 ± 0.27	2.23 ± 0.18	0.24 ± 0.02	3.56 ± 0.32	0.47 ± 0.08	0.06 ± 0.01	0.69 ± 0.11
RGT Siroca	8.13 ± 0.5 b	0.59 ± 0.03	8.05 ± 0.55	4.80 ± 0.79 ab	0.29 ± 0.05	2.98 ± 0.39	2.82 ± 0.75	0.25 ± 0.06	4.30 ± 0.79	0.52 ± 0.03	0.06 ± 0.01	0.77 ± 0.06
Tofina	8.26 ± 0.58 b	0.57 ± 0.03	8.31 ± 0.5	4.27 ± 0.55 b	0.26 ± 0.03	2.85 ± 0.37	3.46 ± 0.68	0.26 ± 0.04	4.70 ± 0.55	0.53 ± 0.05	0.05 ± 0.01	0.76 ± 0.07
Variety (V)	< 0.05	0.112	0.270	< 0.05	0.064	0.898	0.428	0.964	0.497	0.199	0.457	0.145

9.3.2. Controlled environment study: Influence of inoculation and phosphorus addition on nodulation and nitrogen fixation

Inoculation with USDA 110 significantly ($p < 0.01$) increased leaf dry weight and leaf area (Table 9.6). Leaf dry weight, stem dry weight and plant dry weight all significantly ($p < 0.05$) increased following P fertiliser addition; however, root dry weight did not show any significant ($p = 0.900$) response. In the inoculated plants nodule number showed a trend in increase following P fertiliser addition, however this was not significant ($p = 0.060$), with RAU showing no significant differences between P treatments ($p = 0.562$). When examining the inoculated plants, no differences were observed in root or whole plant dry weight, presumably because of the reduced pot size leading to plants becoming pot bound. Interestingly, there was still a trend ($p = 0.060$) in increased nodule number however no significant difference in RAU was observed ($p = 0.562$). This suggests that whilst plant growth is limited by pot size, the availability of additional P increased the allocation of resources to nodule formation, but this was not sustained in an improvement in N_2 fixation processes.

There was a trend ($p = 0.075$) in differences in plant dry weight response to P fertiliser addition depending on inoculation status, with the uninoculated plants showing an increased response (27% increase compared to 4%) to P fertiliser addition (Table 9.6). Uninoculated plants depend only on the low levels of soil available N (Table 9.2) and appeared to be N limited (whilst not quantified, this was observed in pale yellow-green colour at the R1 phase of phenological development). Phosphorus fertiliser addition has potential to stimulate N uptake efficiency, through both the increased assimilation and utilisation of N to maintain N: P stoichiometric homeostasis, increased provision of ATP for N uptake processes, and potential enhanced microbial activities stimulated by P addition (Xia et al., 2023). Whilst root dry weight did not significantly respond to P addition ($p = 0.900$), a slight trend in increase in root dry weight was observed under uninoculated plants ($p = 0.173$). Whilst not quantified in this study we are suggesting that this slight increase in root dry weight may have been related to altered root morphology, such as longer or more branched roots to maximise soil exploration (Rey et al., 2023). This increase in altered root morphology may allow for increased uptake of P added to the system as fertiliser, aiding in the increased plant biomass response to P fertiliser addition in uninoculated plants. The inoculation of soybean with USDA 110 in the second CE study significantly ($p < 0.05$) increases leaf dry weight and leaf area. The inoculation of soybean with USDA 110 allowed for symbiotic N_2 fixation to increase plant N content. The combination of enhanced N supply requiring additional C to maintain C:N ratio, and nodules acting as C sinks provides a demand for greater demand for photosynthetic products, creating a feedback loop leading to increased leaf area and leaf biomass production observed (Table 9.6), to allow for increase rates of photosynthesis (Giller and Cadisch, 1995).

Table 9.6. Plant morphological traits, nodule number, and relative abundance of ureide (RAU) of soybean (Merlin variety) grown under two phosphorus (P) treatments (control and P fertiliser addition) and two inoculation treatments (inoculated with USDA 110 and uninoculated). Individual plants in pots were harvested at the R1 phase of phenological development). Data are means ± SE of 8 replicates. Two-way ANOVA *p* values are displayed.

Source of Variation		Leaf dry weight (g)	Stem dry weight (g)	Root dry weight (g)	Plant dry weight (g)	Leaf area (cm^3)	Specific leaf area ($cm^3\ g^{-1}$)	RAU (%)	Nodule number (No. $plant^{-1}$)
Inoculant	**Phosphorus**								
Uninoculated	Control	0.53 ± 0.04	0.46 ± 0.08	0.66 ± 0.04	1.65 ± 0.1	137.33 ± 7.25	347.82 ± 50.31	-	-
Uninoculated	P addition	0.73 ± 0.03	0.62 ± 0.05	0.72 ± 0.03	2.10 ± 0.1	165.57 ± 10.2	275.47 ± 24.05	-	-
Inoculated	Control	0.79 ± 0.03	0.54 ± 0.04	0.65 ± 0.03	1.98 ± 0.05	163.32 ± 3.67	293.72 ± 15.1	68.98 ± 4.31	53.80 ± 2.79
Inoculated	P addition	0.84 ± 0.05	0.61 ± 0.04	0.62 ± 0.04	2.06 ± 0.11	186.01 ± 7.4	319.73 ± 17.39	72.97 ± 5.22	61.50 ± 2.76
	Inoculant (I)	<0.001	0.622	0.173	0.202	< 0.01	0.920	-	-
	Phosphorus (P)	< 0.05	<0.05	0.900	< 0.05	< 0.01	0.776	0.562	0.060
	I x P	0.101	0.380	0.292	0.075	0.709	0.053	-	-

Table 9.7. Nitrogen derived from fixation (Ndfa) and relative abundance of ureide (RAU) measured using relative ureide assay of both stem and xylem sap and $\delta^{15}N$ natural abundance ($\delta^{15}N$ NA) methods, of soybean grown under contrasting phosphorus (P) treatments in the controlled environment conditions and four varieties of field grown soybeans. Data is displayed as mean ± standard error for all treatments. The controlled environment stem tissue and $\delta^{15}N$ NA data is comprised of 14 replicates, the controlled environment xylem sap data is comprised of 7 replicates, and the field trial data is comprised of 4 replicates. One-way ANOVA *p* values are displayed.

Source of Variation		Stem tissue		Xylem sap		$\delta^{15}N$ NA
		RAU (%)	Ndfa (%)	RAU (%)	Ndfa (%)	Ndfa (%)
Pot	Control	68.98 ± 4.31	111.43 ± 3.34	84.52 ± 2.64	120.03 ± 4.12	62.58 ± 3.1
	Phosphorus addition	72.97 ± 5.22	114.21 ± 4.21	85.92 ± 3.16	122.22 ± 4.93	67.3 ± 3.03
	Phosphorus (P)	0.562	0.611	0.752	0.752	0.288
Field	Merlin	15.12 ± 1.57	55.73 ± 2.57	-	-	31.11 ± 2.53
	RGT Siroca	17.03 ± 0.72	58.78 ± 1.07	-	-	40.65 ± 5.46
	SY Livius	15.47 ± 1.86	56.23 ± 2.9	-	-	38.56 ± 2.16
	Tofina	17.74 ± 3.29	59.17 ± 5.19	-	-	30.27 ± 4.62
	Variety (V)	0.807	0.859	-	-	0.187

9.3.3. Assessment of nitrogen fixation methods

A significant linear regression (R^2 = 0.811, p < 0.001) was observed between Ndfa derived from stem tissue RAU method and δ^{15}N NA methods across both experimental systems (Figure 9.1.a). However, when employing the quadratic and linear functions proposed by Herridge and Peoples (1990), Ndfa was overestimated, with both stem tissue and xylem sap RAU giving Ndfa values greater than 100%, which not only is biologically impossible is almost 200% greater than the Ndfa values recorded using δ^{15}N NA methods (Table 9.7). When the quadratic function proposed by Herridge and Peoples (1990) was employed to the stem tissue RAU and δ^{15}N NA data collected in both the field trial and CE study, the mse (mse = 55.91 and 734.60 respectively) and R^2 value (R^2 = -2.77 and -1.87 respectively) suggested the quadratic function does not accurately capture the relationship observed in the datasets (Figure 9.1). In the field trial, where RAU% were much lower, Ndfa values calculated from RAU stem tissue were also 66.5% greater than those recorded using δ^{15}N NA methods.

When comparing methods for assessing RAU, the xylem sap samples had 19.8% greater RAU than the stem tissue extracts (Table 9.7), with neither showing a significant response to P fertiliser (p = 0.752 and 0.562 respectively). When comparing the RAU xylem sap method for calculating Ndfa with the δ^{15}N NA method, a non-significant linear regression (R^2 = 0.256, p < 0.622) was observed suggesting no correlation between the methods (Figure 9.1). When the linear function proposed by Herridge and Peoples (1990) was employed to the xylem sap RAU and δ^{15}N NA data collected in the CE study, the mse (mse = 464.26) and R^2 (R^2 = -11.53) suggest the linear function does not accurately capture the relationship observed in the dataset (Figure 9.2).

In both experiments, whilst Ndfa derived from the RAU stem tissue method were significantly correlated (Figure 9.1), the expected y = x relationship was not achieved (y = 1.5x + 11.7), suggesting the two methods are not directly comparable. These results suggest that the regression equation for conversion from RAU to Ndfa proposed by Herridge and Peoples (1990) leads to an overestimation of Ndfa, particularly at the higher levels of RAU. The underestimation of stem RAU can occur because of smaller ureide concentration in basal section of stem (Patterson and LaRue, 1983; Rosso et al., 2021), and the reliability of petioles for RAU estimation is dependent on the transport of N solutes, with increased ureide accumulation when shoot N demand is greater (Rosso et al., 2021). Under water deficit, shoot ureide concentrations can also increase, leading to the overestimation of Ndfa (Serraj and Sinclair, 1996). Whilst the plants in the CE study did not show any signs of water stress, the small pot size may have contributed to water deficit increasing shoot ureide concentrations.

The seminal work of Herridge and Peoples (1990) found a trend for the RAU values of early-maturing genotypes to be higher at the same level of δ^{15}N NA Ndfa. In the 30 years since the regression equations were proposed by Herridge and Peoples, soybean breeding has continued to advance, particularly when considering the focus on early maturity genotypes to meet the demand for increased European soybean production. As a result, the early maturity genotypes used in this study, and subsequent studies, may also be outliers from the original conversion equation. Poor agreement between with the published relationships

between RAU and Ndfa by Herridge and Peoples have also been observed in other experimental studies (e.g. (Purcell et al., 2004; Schweiger et al., 2012). As a result, it is suggested that care needs to be taken when employing the equations to determine Ndfa in early maturity soybean, with the requirement for further research being undertaken to re-establish suitable regression equations for these early maturity soybeans.

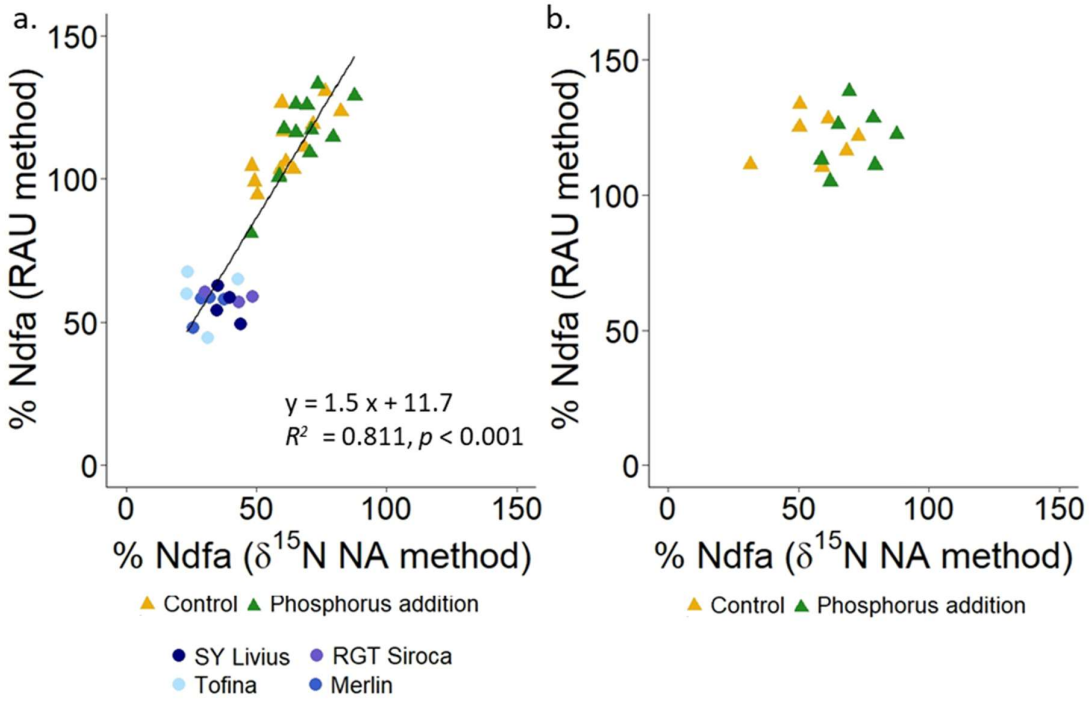


Figure 9.1. The relationship between two methods for quantifying nitrogen derived from fixation (% Ndfa). The x-axis shows Ndfa determined by δ^{15}N natural abundance (δ^{15}N NA) method and the y-axis shows Ndfa determined by relative abundance of ureides (RAU) in **Figure 9.1.a.** stem tissue, and **Figure 9.1.b.** xylem sap. Each data point represents an individual plant response, where RAU% and δ^{15}N NA was measured. Colour represents different treatments, with the green and yellow triangles representing phosphorus fertiliser addition and control treatments respectively in the controlled environment study, and the different shades of blue circles representing the different varieties in the field trial. Linear regressions were fitted where significant, with trend lines displayed on the graphs and equations and R^2 values displayed above.

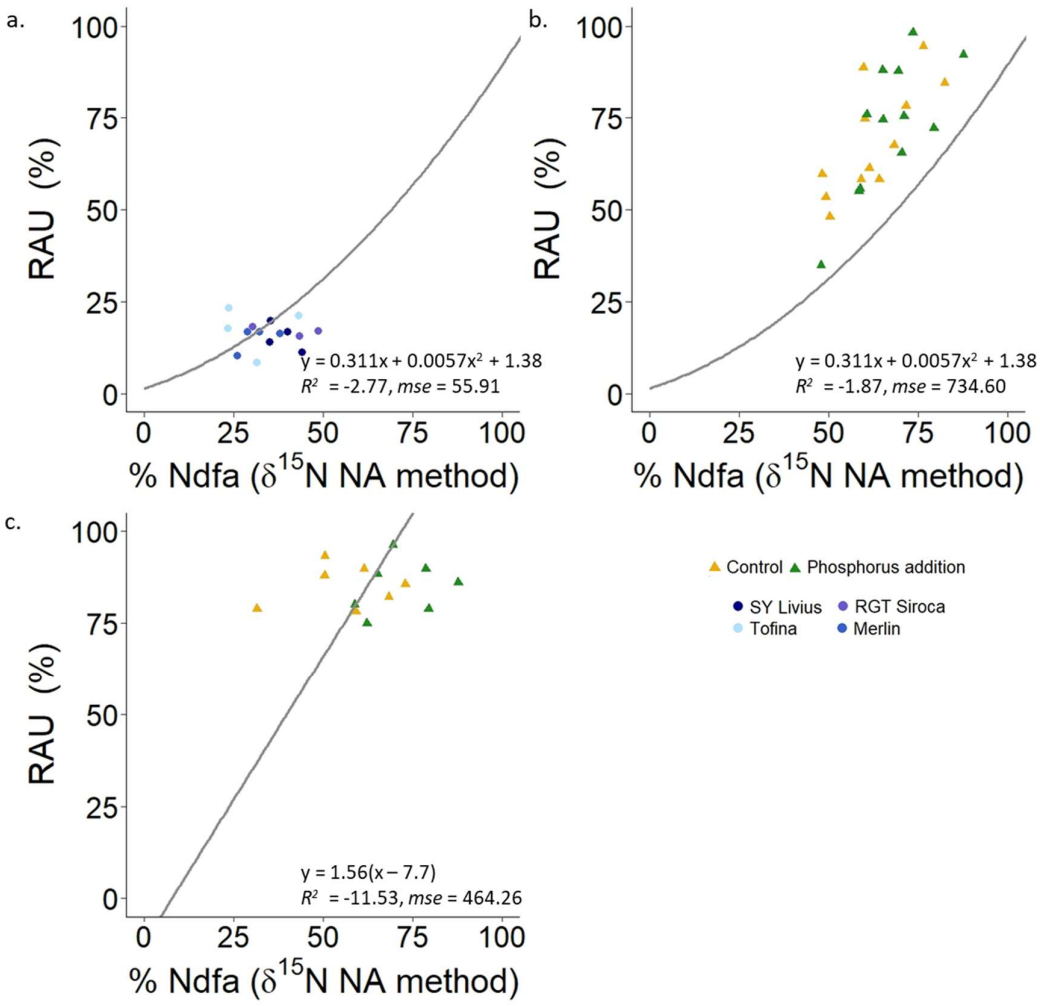


Figure 9.2. Relative abundance of ureides (RAU) measured using relative ureide assay of both stem and xylem sap and nitrogen derived from fixation (Ndfa) measured using natural abundance (δ^{15}N NA) methods. **Figure 9.2.a.** Relative abundance of ureides in stem samples compared to Ndfa measured using δ^{15}N NA of the four varieties of soybean grown in the field trial, **Figure 9.2.b.** RAU in stem samples compared to Ndfa measured using δ^{15}N NA of the soybean grown under contrasting phosphorus (P) treatments in the controlled environment conditions, and **Figure 9.2.c.** RAU in xylem sap samples compared to Ndfa measured using δ^{15}N NA of the soybean grown under contrasting P treatments in the controlled environment conditions. Colour represents different treatments, with the green and yellow *triangles* representing P fertiliser addition and control treatments respectively in the controlled environment study, and the different shades of blue circles representing the different varieties in the field trial. The fitted line is the relationship found by Herridge and Peoples (1990) for vegetative soybean stem tissue ($y = 1.38 + 0.311x + 0.0057x^2$) (Figure 9.2.a and b) and xylem sap ($y = 1.56x – 7.7$) (Figure 9.2.c).

10. Appendix C

Appendix C contains supporting information regarding a preliminary experiment used to inform the experimental design and methodology of Chapters Four and Five.

10.1. Rational

Phosphorus fertiliser addition is required for the process of N_2 fixation to optimise nodule formation and maintaining functional nodule tissue and the provision of ATP (Li et al., 2018). Whilst critical soil P test levels for soybean production have been estimated to be approximately 9 mg P kg-1 Olsen's extractable P, this is dependent on environmental and management factors, and the critical levels for nodulation and N_2 fixation response are less well understood (Culman et al., 2020; Scott et al., 1987). The preliminary work ensures a nodulation and N_2 fixation response following P fertiliser addition can be obtained, particularly because of the challenges associated with sourcing a low-P soil within the UK due to the over-use of P fertiliser within the country leading to the build of legacy P and soil available P concentrations above crop demand.

Due to the growth conditions of Northern England (where most experimental work of this PhD occurred) beyond the climatic extremes of current soybean production and resource limitations, large proportions of experimental work within this thesis have been completed under controlled environment (CE) conditions. Growing soybean under CE conditions compared to field conditions, particularly within the constraints of resources available, has several challenges. Changes in light intensity can lead to differences in leaf morphology, plant dry matter, photosynthetic rate and plant etiolation (Feng et al., 2019; Wu et al., 2017; Yang et al., 2018). Ensuring experimental conditions can be created where the effect of P fertiliser addition can be observed is essential this PhD.

As a result, the aim of this study was to evaluate soybean response under different environmental conditions to ensure successful soybean growth and N_2 fixation response to P fertiliser addition could be quantified in future studies.

10.2. Materials and Methods

Two soybean genotypes (Merlin and RGT Siroca) were grown in a randomised complete block design, with eight replicates per treatment, under two P treatments (control and P fertiliser addition) and two contrasting light intensities. Light intensity was maintained through altering the height of the benches and lights weekly to maintain consistent distances between plant canopy and lights, with canopy PAR measured weekly with a handheld spectral PAR meter, to maintain canopy PAR of 400 and 800 µMol m^{-2} s^{-1}. Four litre pots were filled with a 3:1 mixture of sandy loam topsoil and Leighton buzzard lime free sand, to lower soil nutrient status. When filling pots, granular magnesium and potassium fertilisers were applied to all pots at rates of 0.15 and 0.4 g pot^{-1} respectively (18 and 86 kg ha^{-1} equivalent. Phosphorus fertiliser (single superphosphate, 18 % P_2O_5) was applied at a rate of 0.75 g P pot^{-1}, equivalent to 60 kg P ha^{-1}

to the P addition treatments, with no P fertiliser being applied to the control treatment. Characteristics of the sand: soil mix prior to fertiliser treatment are given in Table 9.2.

Prior to sowing, seeds were first sterilised by immersion in 70% ethanol for 45 s, rinsed in sterile water, immersed in 5% NaHCl for five minutes, then rinsed in sterile water five times. Following sterilisation, seeds were pre-germinated on sterile filter paper. Following successful germination, seeds were selected for uniformity with two seeds being transplanted at 3 cm depth in the centre of 4 l pots. On transplanting, seeds were inoculated with 1 ml *B. japonicum* USDA110 at a density of 1 x 10^8 CFU. USDA110 was previously cultured on MAG agar for 7 days at 30°C. Following successful emergence, at ten days, healthy plants were thinned to one per pot for uniformity. Plants were watered daily to maintain a water holding capacity between 70 & 80%. Plants were grown in a CE room at Lancaster University, with an average day/night temperature 25/18°C and a 16-hour photoperiod (8:00 - 24:00, with a 30-minute ramping period between 8:00 and 8:30). Micronutrients were applied in the form of an adapted N and P free Hoagland's solution (Table 9.3) at a rate of 1 ml l^{-1} of water applied at day ten and biweekly until harvest.

Plants were harvested when soybean reached full flowering (R2) according to average physiological development. At harvest, plants were de-topped and above-ground material was partitioned into leaf and stem. Total leaf area was measured in the controlled environment studies using a leaf area meter (Li-3100 Leaf Area Meter, Li-Cor Inc., Lincoln, Nebraska, USA). Below-ground material was washed to remove any soil, then nodules were removed and counted by hand. Nodules were classified as small (< 2mm diameter) or large (> 2mm diameter) by passing fresh nodules through a 2mm sieve.

10.3. Results

Lower light intensity was found to significantly (p < 0.001) increase plant height by approximately 15.5 cm (Table 10.1). Light intensity did not significantly influence specific leaf area (p = 0.197) or leaf area (p = 0.452), however there was a trend (p = 0.057) in decreased leaf dry weight under lower light intensities. Phosphorus fertiliser significantly influenced all plant morphological traits (Table 3.6; leaf area was significantly (p < 0.001) greater under P fertiliser addition, with specific leaf area significantly (p < 0.05) decreased under P fertiliser addition. Plant dry weight had a significant (p < 0.05) light intensity P interaction, with higher light intensity and P fertiliser addition having significantly greater plant dry weight (p < 0.05) than the other treatments. A significant (p < 0.05) light intensity P interaction was also observed in root dry weight, with higher light intensity and P fertiliser addition having significantly greater root dry weight than the other treatments. Plant dry weight had a significant (p < 0.05) P variety interaction, with Merlin P addition having a significantly (p < 0.05) greater plant dry weight than RGT Siroca and Merlin under control P treatment, and RGT Siroca under P addition having a significantly (p < 0.05) greater plant dry weight than Merlin under control P treatment.

Nodule number increased significantly (p < 0.001) following P fertiliser addition and under high light intensity, with RGT Siroca having significantly (p < 0.05) greater nodule number than Merlin. No significant interactions were observed. Nodule dry weight increased significantly

($p < 0.001$) following P fertiliser addition. Light intensity significantly ($p < 0.001$) affected the proportion of small and large nodules, with significantly ($p < 0.05$) greater percentage of small nodules observed under high light intensities. Merlin had a significantly ($p < 0.05$) greater percentage of large nodules than RGT Siroca, but RGT Siroca had a significantly ($p < 0.05$) greater nodule dry weight. Average nodule dry weight was significantly ($p < 0.01$) greater following P fertiliser addition. Relative abundance of ureides (measured only under higher light intensities) was significantly ($p < 0.01$) greater following P addition compared to control treatment (Figure 10.1).

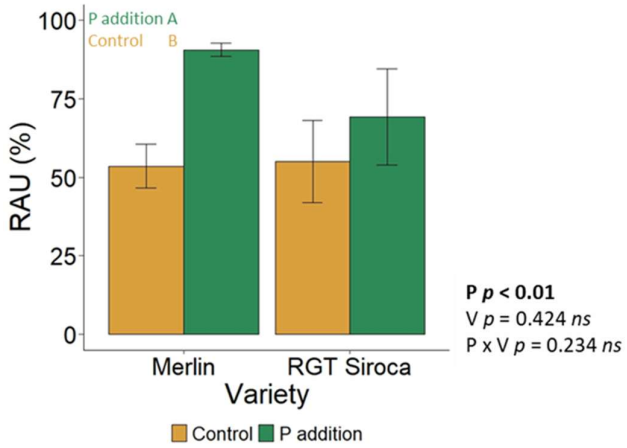


Figure 10.1. Relative abundance of ureides (%) determined from stem extracts (Herridge and Peoples, 1990) of two varieties of soybean (Merlin and RGT Siroca) grown under two phosphorus (P) treatments (control and P fertiliser addition). Data are means ± SE of 8 replicates. Two-way ANOVA *p* values are displayed on the right of the figure, with letters of significance indicating results of Tukeys post-hoc test, where the colour represents the P treatment.

Table 10.1. Plant morphological traits of two varieties of soybean (Merlin and RGT Siroca) grown under two phosphorus (P) treatments (control and phosphorus fertiliser addition) and two light intensities (high and low light intensity). Individual plants in pots were harvested at the R2 phase of phenological development). Data are means ± SE of 8 replicates. Three-way ANOVA *p* values are displayed.

Source of Variation			Height (cm)	Leaf dry weight (g)	Stem dry weight (g)	Root dry weight (g)	Plant dry weight (g)	Specific leaf area ($cm^3\ g^{-1}$)	Leaf area (cm^3)
Variety	**Light Intensity**	**Phosphorus**							
Merlin	Low	Control	67.10 ± 3.16	1.55 ± 0.09	0.94 ± 0.05	1.34 ± 0.29	3.98 ± 0.40	302.06 ± 11.59	464.3 ± 25.09
Merlin	Low	P addition	74.68 ± 4.71	2.24 ± 0.24	1.69 ± 0.24	1.70 ± 0.18	5.97 ± 0.28	259.79 ± 14.39	618.79 ± 54.8
Merlin	High	Control	49.08 ± 2.96	1.82 ± 0.13	1.09 ± 0.11	1.50 ± 0.10	4.6 ± 0.26	276.64 ± 18.7	551.49 ± 46.03
Merlin	High	P addition	58.41 ± 3.61	2.76 ± 0.15	1.78 ± 0.13	2.42 ± 0.15	7.33 ± 0.36	249 ± 16.7	673.36 ± 40.29
RGT Siroca	Low	Control	54.00 ± 5.71	1.45 ± 0.30	0.98 ± 0.04	2.21 ± 0.79	4.76 ± 1.08	319.03 ± 6.48	493.62 ± 60.08
RGT Siroca	Low	P addition	79.53 ± 7.53	1.94 ± 0.12	1.34 ± 0.05	1.33 ± 0.14	4.87 ± 0.28	301.55 ± 22.27	583.07 ± 48.41
RGT Siroca	High	Control	54.90 ± 9.10	1.64 ± 0.25	0.98 ± 0.22	1.46 ± 0.24	4.26 ± 0.72	338.26 ± 89.73	897.55 ± 340.84
RGT Siroca	High	P addition	57.30 ± 4.44	2.29 ± 0.42	1.73 ± 0.15	2.19 ± 0.26	6.62 ± 0.68	399.49 ± 68.31	668.25 ± 76.36
		Light Intensity (L)	< 0.001	0.057	0.224	0.102	< 0.05	0.197	0.452
		Variety (V)	0.942	0.105	0.386	0.788	0.725	0.223	0.343
		Phosphorus (P)	<0.01	< 0.001	< 0.001	< 0.05	< 0.001	< 0.05	< 0.001
		L x V	0.421	0.676	0.670	0.423	0.4355	0.668	0.544
		L x P	0.315	0.461	0.656	< 0.05	< 0.05	0.697	0.779
		V x P	0.421	0.069	0.446	0.072	< 0.05	0.637	0.519
		L x V x P	0.086	0.429	0.316	0.189	0.098	0.239	0.480

Table 10.2. Nodulation response of two varieties of soybean (Merlin and RGT Siroca) grown under two phosphorus (P) treatments (control and P fertiliser addition) and two light intensities (high and low light intensity). Individual plants in pots were harvested at the R2 phase of phenological development). Nodule number, dry weight, size category (small, <2 mm or large, ≥2 mm), specific dry weight (nodule dry weight normalised for root dry weight, g g^{-1}) and average nodule dry weight (calculated by nodule dry weight divided by nodule number). Data are means ± SE of 8 replicates. Three-way ANOVA *p* values are displayed.

Variety	Source of Variation: Light Intensity	Phosphorus	Nodule number	Small nodules (%)	Large nodules (%)	Nodule dry weight (g)	Average nodule dry weight (mg $nodule^{-1}$)
Merlin	Low	Control	55.0 ± 4.9	41.3 ± 7.8	58.7 ± 7.8	0.14 ± 0.03	2.9 ± 0.8
Merlin	Low	P addition	80.6 ± 7.3	29.1 ± 4.6	70.9 ± 4.6	0.25 ± 0.02	3.2 ± 0.4
Merlin	High	Control	102.8 ± 13.8	53.6 ± 6.2	46.4 ± 6.2	0.17 ± 0.02	1.9 ± 0.3
Merlin	High	P addition	149.6 ± 18.2	53.7 ± 3.7	46.3 ± 3.7	0.31 ± 0.04	2.1 ± 0.2
RGT Siroca	Low	Control	82.4 ± 4.2	45.8 ± 6.2	54.2 ± 6.2	0.15 ± 0.25	1.8 ± 0.3
RGT Siroca	Low	P addition	150.8 ± 9.1	44.0 ± 3.7	56.0 ± 3.7	0.38 ± 0.06	2.5 ± 0.4
RGT Siroca	High	Control	109.3 ± 11.0	65.9 ± 2.7	34.1 ± 2.7	0.21 ± 0.05	1.8 ± 0.4
RGT Siroca	High	P addition	162.6 ± 25.5	55.9 ± 4.7	44.1 ± 4.7	0.44 ± 0.07	3.0 ± 0.4
		Light Intensity (L)	< 0.001	< 0.001	< 0.001	0.094	0.123
		Variety (V)	< 0.05	< 0.05	< 0.05	< 0.05	0.222
		Phosphorus (P)	< 0.001	0.087	0.087	< 0.001	< 0.01
		L x V	0.734	0.734	0.734	0.769	0.250
		L x P	0.789	0.789	0.789	0.813	0.505
		V x P	0.964	0.964	0.964	0.082	0.167
		L x V x P	0.172	0.172	0.172	0.768	0.327

10.4. Discussion

One of the objectives of this study was to evaluate soybean growth response under contrasting light conditions to ensure suitable conditions were selected for future growth in CE conditions for this thesis. Light availability is one of the major environmental parameters regulating plant physiology, due to its role as an energy source in photosynthesis and signal to activate and regulate many other processes (Devlin et al., 2007). Under the lower light intensity, soybean plant height significantly increased (Table 10.1.), agreeing with previous studies that found reduced canopy PAR leads to increases in soybean internode length, increasing plant height (Xu et al., 2021). As a result, canopy PAR was maintained at 800 µMol m^{-2} s^{-1} in future experimental work, to reduce plant etiolation and replicate environmental conditions closer to the field optimum.

In the CE study reported in Chapter Three and Appendix B, the smaller 0.75 l pot size required for use in the pressure chamber led to reduced growth compared to the bigger 4 l pots used in the first. This is thought to be due to the combination of plants quickly becoming pot ground leading to difficulties maintaining water and nutrient availability, with plants though to be to be under stress due to early flowering being observed. When looking at the second CE study, nodulation and RAU was also reduced, despite other environmental and management conditions being consistent. This implies that the reduced plant growth observed has led to a reduction in resource allocation to nodules, reducing N_2 fixation rates, or the reduced demand for N_2 fixation due to reduced N requirements because of lower plant growth. The low nodulation and N_2 fixation results observed in the field trial suggests low levels of rhizobial activity and low inoculation success (Table 9.4). As a result, in CE studies were inoculated with USDA 110 at similar CFU. This appeared to be much more successful, with nodule number in the rage of 50 – 180 across both CE studies (Table 10.2).

To achieve a low soil available P status, something uncommon of UK agricultural conditions due to high legacy P stocks, the soil used in this experiment was mixed with a commercial Leighton buzzard lime free sand. As a result, the sandy soil had a low nutrient status and needed the application of other micro- and macro-nutrients to ensure no further nutritional stress, and the sandy soil, coupled with the growth in pots, meant low water holding capacity so a regular watering regime needed to be implemented to ensure no water stress was inflicted. In future experiments, as in these pot trials, plants will be watered daily to maintain WHC of 70 – 80%, with macro nutrients applied as granular fertiliser at planting, and micro-nutrients applied bi-weekly as an adopted N and P free Hoaglands solution. Future experimental analysis will include the analysis of leaf tissue to ensure no unwanted plant macro- or micro- nutrient deficiencies were observed (Section 5.2.3.).

10.5. Summary of methods for future experimental work

The results of this study have been primarily reported to inform and guide future experimental work undertaken within this thesis. The following environmental conditions and experimental designs will be applied to experimental work in chapters four and five: CE light positioning will be altered throughout the growth cycle to maintain canopy PAR at 800 µMol m^{-2} s^{-1}; plants will be inoculated with USDA 110 at 10^8 CFU, P addition treatments will receive

P fertiliser at a rate equivalent to 60 kg P ha^{-1}. Plants will be grown in a 3:1 sand: soil mix in 4 l pots, watered daily to maintain WHC of 70 – 80%, with macro-nutrients applied as granular fertiliser at planting, and micro-nutrients applied bi-weekly as an adopted N and P free Hoaglands solution.

Due to difficulties in measuring xylem sap RAU because of the smaller pot size and resource constraints limiting the ability to use the NA technique, all future analysis will be completed on stem tissue RAU and all future work within this thesis will only present N_2 fixation as either RAU or total shoot N mass. No conversion between RAU and Ndfa will be made due to the overestimation in Ndfa observed using the regression equations proposed by Herridge and Peoples (1990), discussed further in Chapter Three.