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Integrating ecological stoichiometry to understand nutrient limitation and potential for competition in mixed pasture assemblages

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Full Title:	Integrating ecological stoichiometry to understand nutrient limitation and potential for competition in mixed pasture assemblages
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Author Comments:	Dear Editor,

	<p>We enclose herewith a manuscript entitled 'Integrating ecological stoichiometry to understand nutrient limitation and potential for competition in mixed pasture assemblages' for consideration for publication in the Journal of Soil Science and Plant Nutrition.</p> <p>Please excuse the delayed response in providing the revised document, we had one co-author away for the month on vacation and wanted to ensure that edits and final approval were able to be completed by all prior to resubmission.</p> <p>We hope that the revised version is to your satisfaction and we thank you for your, and the reviewers careful comments towards the improvement of the manuscript.</p>
<p>Response to Reviewers:</p>	<p>Dear Dr. Barra,</p> <p>We sincerely wish to thank the expert reviewers and editor for their clear and thorough analysis of our manuscript, and we hope that the following responses and revisions to the manuscript will be satisfactory for its inclusion in The Journal of Soil Science and Plant Nutrition.</p> <p>Herein, we address each of the specific reviewer's comments as requested. Thank you again for your time and consideration of our manuscript.</p> <p>Kind regards, Dr. Kirsten Ball</p> <p>Comments to the author (if any):</p> <p>Reviewer #1: The authors sufficiently considered my comments. The revisions by the authors strengthen the interpretation and the discussion of the experimental outcomes. The manuscript provides a good starting point for further research and for data comparison. I have no further comments on the manuscript.</p> <p>Reviewer #3: This manuscript has improved a lot after first round of revision, I suggest minor revision before accept.</p> <p>1. In the manuscript, the author use model mixed-pasture system to represent agricultural system, which may not be very property. Author response: Unfortunately, we are having difficulty understanding this comment from reviewer 3 and therefore have not addressed it. If the editor has a concern about the use of the term 'model' for the study system, we can correct it. However, we want to make it clear to the reader that these species were selected because they are common in mixed pastures, both at the species level but more to the point, as plant functional types. Hence the term "model" mixed-pasture system.</p> <p>2. the subtitle in the result and discussion section should not be written into sentences, like Nutrient addition and cultivation type both positively influence aboveground biomass, etc. Author response: Thank you for this comment which we have addressed by shortening the subheadings throughout the results section. However, we do not agree that subheadings should be changed from statements in the results section, as this structure serves to form the 'story setup' for each paragraph. We have therefore left the discussion subheadings as is.</p> <p>3. the conclusion is too long, and references should be discussed in other sections. Please state your conclusions. Author response: Thank you for this comment. We have considerably shortened the conclusion section and focused on the broader implications of the study, that is, the importance of considering plant trait variation in the context of stoichiometric homeostasis to understand nutrient dynamics.</p> <p>Editor Comments.</p> <p>- Please provide an adequate response to each reviewer's comment and submit a list of responses to the comments indicating specifically in which lines the improvements were done, or explaining why they were not considered. Furthermore, I inform you that due to JSSPN policies, we do not accept changes in the authorship of the manuscript</p>

once the peer review process has begun.

- Scientific names must be written in italics, even in references. Please correct it.
Author response: Thankyou. This has been done.

- Please, remember that tables and figures must be self-explanatory. That is, statistics and abbreviations used must be clearly explained.

Author response: Thank you for this comment and we apologize this was not sufficiently addressed in the previous version. We have now added the expanded explanation for statistical notation as opposed to abbreviations.

- The conclusions section should not be a summary of your study or an extension of the discussion. The conclusions section should illustrate the mechanistic links of findings obtained under applied treatments.

Author response: Thank you for this comment. We have removed the reiteration of the study results and focused on the broader implications of the study, that is, the importance of considering plant trait variation in the context of stoichiometric homeostasis to understand nutrient dynamics.

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Integrating ecological stoichiometry to understand nutrient limitation and potential for competition in mixed pasture assemblages

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Abstract

Purpose: Much is known about growth and nutrient uptake traits and ecological stoichiometry in natural systems. However, these concepts have been comparatively understudied in agricultural systems despite their potential to infer nutrient limitation and interspecific resource competition.

Methods: This study established a model mixed-pasture system to assess tissue C:N and C:P stoichiometry and above ground biomass (AGB) in a grass (*Phalaris aquatica*) and legume (*Trifolium vesiculosum*) under factorial inputs of high and low nitrogen (N) and

phosphorus (P), in monoculture and mixture. Due to inherent trait diversity, we expected grass and legume growth, shoot vs root stoichiometry and N:P homeostasis to differ in response to nutrient limitation and between monoculture and mixture.

Results: Grass AGB was greater with N addition and in mixture, and legume AGB was decreased by N but increased by P, more so in mixture. Nutrient limitation in grass was determined *via* a strong coupling of growth with shoot stoichiometry, by which AGB decreased and C:N increased under N limitation. Legume growth was not correlated with tissue stoichiometry, but potential for growth limitation by N and P was detected *via* increased shoot C:N under low N and P, and C:P under low P. Legume shoot N:P was more homeostatic than grass, and grass shoot N:P homeostasis was greater in mixtures than in monocultures.

Conclusions: Integrating ecological stoichiometry alongside trait-based ecology is a useful tool for predicting how fertiliser management may affect nutrient balance and species dominance in mixed pasture agroecosystems.

Keywords

Agriculture, Forage, Facilitation, Fertilization, Homeostasis, Flexibility, C:N:P ratios

Introduction

Plant traits determine individual requirements for nitrogen (N) and phosphorus (P), and ecological stoichiometry regulates the distribution of the elements carbon (C), N and P in ecosystems (Sternner, 2002). Therefore, understanding traits such as growth rate, and nutrient allocation between roots and shoots that influence, and are influenced by, plants' relative N and P requirements make it possible to infer the potential effects of stoichiometric imbalances

on primary productivity and community dynamics (Meunier et al., 2014). Organisms are considered to exist on a continuum from strict homeostasis to ‘flexibility’ regarding their ability to regulate internal nutrient composition, relative to their soil (Sterner, 2002). A loosely coupled tissue:substrate stoichiometry is homeostatically strict; in contrast, tissue stoichiometry tightly coupled to that of the substrate is considered homeostatically flexible (**Fig. 1**). Relative stoichiometric flexibility in plants is partially attributed to their ability to allocate C, N and P towards different physiological processes and to take up elements in excess of requirements for growth (luxury consumption or nutrient conservation) (He et al., 2009; Hessen et al., 2004). Given that plant growth and nutrient use traits determine their degree of N:P homeostasis, fast-growing, nutrient-acquisitive species such as grasses are usually more homeostatically ‘flexible,’ whereas slower-growing more nutrient-conservative species like legumes are likely more homeostatic (Guo et al., 2017; Poorter et al., 2014; Reich, 2014). Although much is known about how N and P addition affects primary productivity in individual crops, less is understood from a stoichiometric perspective about how plant-plant interactions and nutrient availability combine to influence stoichiometric ratios, and how this information may be used to better our understanding of agricultural nutrient limitation.

In terrestrial systems, nutrient limitation is often referred to as a single or multiple nutrient constraint leading to reduction in net primary productivity (NPP) relative to potential NPP under non-limiting conditions (Bracken et al., 2015). In individual plants, diagnosis of single nutrient limitation has been determined *via* decreased growth rate coupled with increased C:nutrient tissue ratio (Ågren, 1988; Ågren, 2004; Droop, 1973); however, C:N:P ratios and growth relationships can differ significantly among species (Elser et al., 2003). Higher tissue N:P has been reported in slower growing species and is often accompanied by luxury

consumption of nutrients independent of a growth response (Chapin, 1980); conversely faster-growing species have a tendency to exhibit lower tissue N:P (Ryser and Lambers, 1995), with a biomass stoichiometry more tightly coupled to substrate N:P (Mendoza et al., 2016a). In cropping systems, it is common to cultivate slow-growing species like legumes, which have higher requirements for P, alongside faster-growing, more N-demanding species such as grasses (Graham and Vance, 2003; Haynes, 1980). This complementary planting is successful partly because legumes use P to enable biological nitrogen fixation (BNF) (Evers, 1982; Mendoza et al., 2016a) which can facilitate the growth of grasses and improve forage quality (Peoples et al., 2015). Moreover, co-planting can up-regulate BNF and improve legume yield (Ledgard et al., 1992; Nyfeler et al., 2011).

To deal with fluctuations in nutrient availability, higher plants have developed a strategy of allocating carbon and nutrients to different organ systems to reflect changing resource requirements (Enquist and Niklas, 2002). Termed ‘biomass partitioning’, the strategy is usually aimed at acquiring the most limiting resource (Chapin, 1991). Plant growth depends on a functional balance between the transport of photosynthate carbon from shoots to roots, and nutrients (including N and P) from roots to shoots (Thornley, 1991). Stoichiometric control over biomass partitioning is important to understand in agricultural systems as differential allocation of nutrients between roots and shoots can be species dependent (Warembourg et al., 2003), decoupled from growth (Gleeson and Tilman, 1994; Hilbert, 1990) and can affect the return of nutrients to the soil (Amato et al., 1984; Johnson et al., 2007). As plant traits drive the relative requirements of N and P among plant functional groups (Elser et al., 2003), and nutrient limitation and community interactions jointly influence nutrient uptake and primary productivity (Ågren, 2008; Ågren, 1988; Ågren, 2004), differential root:shoot biomass and nutrient partitioning among species within functionally

diverse plant communities can be an indicator of nutrient competition (Ashton et al., 2010; Nasto et al., 2017; Warembourg et al., 2003).

Although C:N and C:P stoichiometry has been used to determine optimal N:P supply ratios for individual pasture species (Agnusdei et al., 2010; Duru and Ducrocq, 1996), comparatively little is known about the stoichiometry of nutrient limitation in mixed pastures under different N:P supply ratios. To better understand these dynamics, our study established a common pasture grass and a legume species in a controlled glasshouse study under factorial combinations of low (L) and high (H) inputs of N and P and examined growth and tissue stoichiometry in roots and shoots to identify nutrient limitation and potential for nutrient competition in mixed pastures. We combined a conceptual understanding of plant growth and nutrient use traits with ecological stoichiometry to establish our predictive framework. We hypothesised that:

- 1) The faster-growing, more nutrient-acquisitive grass would be more homeostatically flexible, with AGB responding positively to N addition and cultivation with a legume.
- 2) The grass would allocate nitrogen to shoots to maximise growth, and therefore N limitation in grass may be determined *via* increases to shoot C:N ratios and reductions in AGB. Competition for N would decrease between grass monoculture and mixture, but competition for P may increase (**Fig. 2**).
- 3) The slower-growing, more homeostatic legume would be positively influenced by P addition owing to the latter's role in biological nitrogen fixation (BNF), and by cultivation with grass *via* up-regulation of BNF. Therefore, competition for P in legume would increase between monoculture and mixture (**Fig 2**).

- 4) The legume is likely to maintain a more homeostatic shoot N:P, whereas root C:N and C:P may be more flexible, owing to increased P allocation towards roots to support nodule development. Potential for nutrient limitation in the legume may be detected *via* increases to root C:P.

Materials and methods

Experimental design

Our experiment was conducted from the 28th of October 2018 in a greenhouse maintaining natural average temperatures of 27°C/16°C on a day/night cycle and an average day length between 07:00 – 19:00 under natural light, for 70 days (Ball et al., 2020). The 70-day growth cycle was chosen to capture the ideal temperature range for germinating and growing both species while avoiding potential for summer dormancy with increasing temperatures (Brar et al., 1991; Watson et al., 2000). We implemented four fertiliser treatments: two levels of nitrogen (LN and HN) and phosphorus (LP and HP) addition (LNLP control, HNHP, HNLP and LNHP) on a grass (*Phalaris aquatica*) and a legume (*Trifolium vesiculosum*) species grown in monoculture and mixture. These species were chosen as *Phalaris* is commonly found in pasture systems globally and is commonly sown with *Trifolium* species (Lavergne and Molofsky, 2004, 2006; Watson et al., 2000). Therefore, we considered the species chosen to represent a ‘model’ system. 10 replicates of 12 treatments resulted in (n = 120) pots. Seeds were supplied by Heritage Seeds Australia, along with the appropriate group C rhizobial inoculant required for *T. vesiculosum*. The experimental design was obtained using CycDesign (Whitaker et al., 2002) and randomized using the `dae` package in R (Brien, 2018; R Core Team, 2020) (**Fig. S1**), (Ball et al., 2020).

Growing conditions

Three kg (dry weight) of pasteurized, unfertilised potting mixture (0.33 sand:0.33 clay loam:0.33 coco peat by volume) at pH 6.3 was potted into 198 mm diameter x 149 mm high (4587 cm³) pots, with drainage holes seated on a 200 mm round dish to retain water and nutrients. Eight seeds were planted in each pot, leaving two seedlings on each half of the pot after thinning (Day 16). In mixture, one half (pot) comprised grass and the other half legume. On day 16, to promote microbial activity and nodulation we added a field soil microbial wash of 1 g of field soil in 100 ml of DI water and molasses (ratio of 100:1 (ml) with the recommended amount of Group C rhizobial inoculant to obtain ~ 3,000,000 rhizobia. Plants were watered once daily, and soil water content was maintained at field capacity (22% (w/w) gravimetric water content) by watering to weight.

Fertilisation

Nitrogen (N) and phosphorus (P) in the forms of ammonium nitrate (NH₄NO₃) and a pH 6.3 balanced mixture of disodium phosphate (Na₂HPO₄) and sodium dihydrogen phosphate (NaH₂PO₄) were prepared in 100ml of DI water. Nutrients were added on a dry-weight, mg kg⁻¹ of soil basis. The low N-low P (LNLP, control) treatment nutrients (33 mg N, and 11 mg P, N:P ratio; 3:1; this translates to 30.56 kg/ha N, 10.18 kg/ha P) were added to all pots on day 16. On day 35, nutrients were added to increase the total amount of N and/or P to desired treatment levels. In total, for the high N-high P (HNHP) treatment, we added 99 mg N and 33 mg P (N:P ratio 3:1); for the HNLP treatment, we added 99 mg N and 11 mg P (N:P ratio 9:1); and for the LNHP treatment, (33 mg N and 33 mg P, N:P ratio; 1:1). Pots received macro- and micronutrients at the following rates (mg kg⁻¹ dry soil): K₂SO₄, 75; CaCl₂.2H₂O, 75; MgSO₄.7H₂O, 45; CuSO₄.5H₂O, 2.1; ZnSO₄.7H₂O, 5.4; MnSO₄.H₂O, 6.4; CoCl₂.6H₂O,

0.33; Na₂MoO₄·2H₂O, 0.18; H₃BO₃, 0.3 and FeEDTA, 0.4. Plant available N and P were determined in plant-free pots as described below. Results are detailed in supplementary materials (**Table S1**).

Final harvest

On day 70, all above- and belowground shoot biomass (AGB, BGB) was harvested and soil samples collected to determine total AGB, shoot and root nutrient concentrations and soil nutrients (N and P). AGB was determined by separating the plants between the two sides of the pot above- and belowground, washing the soil from the roots and cutting at root crown level to separate above and belowground biomass. Biomass was dried at 70°C and weighed; the reported AGB is the total weight of the two individual plants from each side of the pot (n = 240) (**Table S2**). We have reported BGB in the supplementary materials (**Table S3**) however we do not discuss total BGB as part of the results as we were unable to separate total root biomass at the pot level by species, and the plants became root-bound towards the end of the experiment. Root nutrient concentrations were obtained from attached root samples to ensure species-level separation. All plants were viable for harvest, except two half pot replicates in the grass only LNLP control treatment which did not survive.

Soil and shoot nutrient analyses

Soil extractable N was determined by shaking 40 ml of 2 M potassium chloride (KCl) solution with 4.0 g soil (< 2 mm) at 170 rpm for 1 hour and then filtering through a 2.5 µm ashless filter (Grade 42, Whatman PLC, Kent, U.K). Extractable P was determined by mixing 4 g soil in 40 ml of 0.5 M NaHCO₃ and shaking for 16 hours (Olsen, 1954). Soil extracts were stored at -20 °C until colorimetric analysis in a discrete analyser (AQ2, SEAL

Analytical, Ltd., Milwaukee, WI USA and EPA135 method). For total carbon (C) and N shoot and root nutrients, a subsample (~3 g) of biomass from each plant was finely ground and homogenised with an MM 400 mixer mill (Retsch, GmbH, Haan, Germany) and an approximately 5 mg subsample was taken for combustion analysis using an Elementar Vario El Cube Carbon/Nitrogen analyser (Elementar Analysensysteme GmbH, Langenselbold, Germany). Phosphorus concentration of tissue samples was obtained after digesting ~ 55 mg of plant material in concentrated H₂SO₄ and H₂O₂ in a microwave digester, and colorimetric analysis after an ammonium molybdate reaction (Crous et al., 2015). Measurement error was suspected in two replicates for total C, N and P; one in the HNLP legume monoculture and one in the LNHP grass monoculture. These were removed from the analysis.

Statistical analysis of growth and tissue nutrient ratios

To compare AGB and tissue nutrient ratios between treatments a linear mixed-model analysis was performed using “ASReml-R” (Butler et al., 2009) and “asremlPlus”(Brien, 2019) packages within R (R Core Team, 2020). The tissue nutrient ratios were natural log transformed. The linear mixed model included terms for the treatment differences, spatial effects, and residual error variation. It was of the following form:

$$\mathbf{y} = \mathbf{X}_t\boldsymbol{\tau} + \mathbf{X}_s\boldsymbol{\beta} + \mathbf{Z}\mathbf{u} + \mathbf{e}$$

where \mathbf{y} is the response vector of values for the trait being analysed; $\boldsymbol{\tau}$ is the vector of fixed treatment effects; $\boldsymbol{\beta}$ is the vector of fixed spatial effects; \mathbf{u} is the vector of random effects; and \mathbf{e} is the vector of residual effects. \mathbf{X}_t , \mathbf{X}_s and \mathbf{Z} are the design matrices corresponding to $\boldsymbol{\tau}$, $\boldsymbol{\beta}$ and \mathbf{u} respectively. The fixed-effect vector $\boldsymbol{\tau}$ was partitioned as $\boldsymbol{\tau}^T = [\mu \quad \boldsymbol{\tau}_{Sp}^T \quad \boldsymbol{\tau}_G^T \quad \boldsymbol{\tau}_L^T]$, where μ is the overall mean; $\boldsymbol{\tau}_{Sp}^T$ incorporates the two species main effects; $\boldsymbol{\tau}_G^T$ contains parameters for the 3 main effects, the 3 two-factor interactions and the three-factor interaction

of the factors cultivation type, nitrogen and phosphorus for the grass (G) species; and τ_L^T contains the same parameters for the legume (L) species. The fixed-effect vector β was partitioned as $\beta^T = [\beta_R^T \beta_{Si}^T \beta_H^T]$, where the β subvectors correspond to the effects of replicates (R), greenhouse sides (Si, east or west) and pot halves (H, east or west) that capture systematic spatial variation within the greenhouse. The random-effects vector u was partitioned as $[u_{R:M} u_{R:M:P}]$ where $u_{R:M}$ is the vector of main-unit (M) random effects within each replicate (R) and $u_{R:M:P}$ is the vector of random effects for pots (P) within each main-unit (M). The residuals e were assumed to be normally distributed with their variance allowed to vary with both species and nitrogen. For each trait, residual likelihood ratio tests with $\alpha = 0.05$ were used to determine whether the variance model can be simplified by removal of the nitrogen level variance difference and/or species variance difference. The model was modified to reflect the results of these tests and residual-versus-fitted values plots and normal probability plots confirmed that model assumptions were met. Wald F-tests at $\alpha = 0.05$ were conducted for the fixed effects within each species to determine a model for describing how cultivation type, nitrogen and phosphorus affect the response for each species. Testing began with the three-factor interaction for a species and, only if it was not significant, proceeded to test the two-factor interactions; the main effects were only tested if that factor had not occurred in a significant interaction. Estimated marginal means were calculated, along with least significant differences for ($\alpha = 0.05$) [LSD(5%)]; they were back transformed from the logs for the tissue nutrient ratios.

Calculation of C:nutrient ratio and growth relationships

The 'lm' function from Base R (R Core Team, 2020) was used to determine the relationship between tissue C:nutrient values and AGB (g) by species and cultivation type. Predictor and

response variables were natural log transformed, and all model assumptions of residual normality and homoscedasticity were met.

Calculations of relative N:P homeostasis

Homeostatic coefficients (H) were calculated from the inverse slope of the line of log-tissue N:P as a response of log-substrate N:P ($H_{N:P}$) (Sterner, 2002). Higher values of H can indicate that tissue N:P is more loosely coupled with substrate N:P (homeostatic), and lower values indicate that tissue N:P is more tightly coupled with substrate N:P (flexible) (Ågren, 2008; Ågren, 2004; Elser and Urabe, 1999). We tested for significant differences in the degree of homeostasis between species, cultivation type and biomass components using a Fisher's r correlation to z-score transformation (Wilcox and Muska, 2002). The correlation coefficient values (r values) were transformed into Fishers Z-scores using **equation 1**.

$$z = 0.5[\ln(1 + r) - \ln(1 - r)] \quad (1)$$

Then, the z scores were compared and analysed for statistical significance accounting for the individual group means and standard deviations and by examining the observed z-statistic (**equation 2**). At $\alpha = 0.05$, our reported z-statistics were determined significant at ± 1.96 .

$$Z_{\text{observed}} = (z_1 - z_2) / \sqrt{[(1 / n_1 - 3) + (1 / n_2 - 3)]} \quad (2)$$

Results

Aboveground biomass responses to cultivation and nutrient addition

In grass, AGB was increased by an interaction between N treatment and cultivation type ($p < 0.001$; **Fig 3a**). Under high N in mixture, grass biomass was ~35% greater than when grown in monoculture. The means and standard errors for the observed AGB data are shown in Table S2.

Legume AGB was increased individually by P treatment ($p < 0.001$; **Fig 3a**) and by an interaction between cultivation type and N treatment ($p < 0.05$; **Fig 3a**). Legume biomass was ~50% greater under P fertilization for both cultivations. In monoculture, N had no effect on legume AGB, but, in mixtures, legume AGB was 36% greater under low N for low P and 25% greater under low N for high P. The best performing combination was legume in mixture with low N and high P.

Relationships between biomass responses and biomass C:N stoichiometry

The means and standard errors for the observed shoot C:N and C:P data are in **Table S4**. In grass, there was a significant relationship between shoot C:N and AGB ($p < 0.001$), where shoot C:N was inversely related to biomass. The slopes differed between monoculture ($r = -0.65$) and mixture ($r = -0.72$), ($z\text{-crit} = 4.32$, **Fig. 4, Table S5**). There were no significant relationships between growth and shoot C:N in legume. Similarly, shoot C:P, root C:N or C:P were not significantly correlated with growth in either species (**Table S6**).

Treatment influences on biomass C:N and C:P stoichiometry

Grass shoot C:N was influenced by N fertilization and an effect of cultivation type, but the effects of the two were independent ($p < 0.001$ for both, **Fig 3b**, **Table S7**). Under low N, grass C:N was 98% greater than under high N, and in monoculture was 32% higher than in mixture. Legume shoot C:N was affected by an interaction between N and P treatment ($p < 0.05$, **Fig 3b**), where under low N and P, legume shoot C:N was at least 10% greater than all other treatments.

For grass shoot C:P, there was an interaction between N and P treatment ($p < 0.001$, **Fig 3c**), and an effect of cultivation type ($p < 0.05$, **Fig 3c**). In both cultivations, grass shoot C:P was greatest under high N and low P and there was no significant effect of P under high N ($p < 0.05$). Monocultures had 14% higher C:P ratios than mixtures. Legume shoot C:P ratios were influenced by P treatment ($p < 0.001$, **Fig 3c**). Legume shoot C:P was highest in low P treatments.

The means and standard errors for the observed root C:N and C:P data are presented in **Table S8**. Grass root C:N revealed a three-way interaction between cultivation type and N and P treatment ($p < 0.05$; **Fig 5a**, **Table S9**). Under low N and P, grass root C:N was significantly higher in monocultures. The same relationship was present under high N and P. Legume root C:N was influenced by P treatment ($p < 0.05$; **Fig 5a**), and by an interaction between cultivation type and P treatment ($p < 0.05$, **Fig 5a**). Grass root C:P was affected by N fertiliser ($p < 0.05$; **Fig 5b**, **Table S8 & S9**), being higher without N addition. Legume root C:P was influenced by both N and P treatment ($p < 0.01$, **Fig 5b**, **Table S9**); for both monoculture and mixture; under low N and high P legumes accumulated the greatest amount of P in roots, ~

80% more than the low N and low P treatment. Under high N treatments, legumes accumulated an intermediate amount of P in roots, regardless of cultivation type.

Homeostasis responses between species and cultivation type

Shoot N:P homeostasis differed between grass and legume ($\alpha = 0.05$, $z\text{-crit} = 1.96$, **Fig 6**, **Table S5**), with legume being more homeostatic ($z\text{-crit} = 2.53$). Grass shoot N:P was more homeostatic in mixture than monoculture ($z\text{-crit} = 2.90$). Root N:P homeostasis did not differ significantly between species ($z\text{-crit} = 0.73$), or within species between cultivation types (**Table S5**). Between roots and shoots when all species and cultivation types were combined, the trend was for roots to be more homeostatic than shoots ($z\text{-crit} = 2.47$).

Discussion

Ecological stoichiometry is a useful tool to infer potential for nutrient limitation in mixed cultivations

As anticipated, the faster-growing, more nutrient-acquisitive grass species responded positively to N addition, and when cultivated with a legume; the slower-growing, more nutrient-homeostatic legume species responded positively to P addition and growth in mixture. Moreover, the grass had a faster relative growth rate than the legume, and this effect was augmented in mixed cultivation (Ball et al., 2020). It is important to note that enhanced growth of grass under N addition in mixture may be attributed to a greater availability of N for two, versus four, grass plants and thus may not be due to facilitation. In order to determine whether facilitation occurred, studies of ^{15}N isotopes would be required (Chalk and Ladha, 1999). Traits like growth rate and nutrient acquisitiveness are important indicators of potential primary productivity (Ansquer et al., 2008), as well as being valuable for comparing productivity between species, assuming that interspecific trait variation is greater than intraspecific variation (Siebenkäs et al., 2015). Arguably, these plant traits are highly plastic within species and thus may be considered unreliable for the purpose of predicting potential NPP, but we assumed that trait variation between grasses and legumes would be greater than that occurring within species. Therefore, examination of plant growth alongside resource allocation traits may be highly informative to determine nutrient limitation in mixed pastures.

Grass growth is intrinsically related to C:N stoichiometry

We observed grass shoot C:nutrient ratios decreasing in response to nutrient addition and when grown with a legume, and this pattern was correlated with increased AGB. Increased

concentrations of N in grass shoots coupled with higher AGB may be due to the high requirement for N in photosynthesis, as high-leaf N content has been linked to fast-growth rates (Freya and Peter, 2017; Wright et al., 2004). There is evidence to indicate species that are stronger competitors for a nutrient require less of it to grow (Elser et al., 2003; Sterner, 2002), and while legumes have higher leaf N content than grasses, grasses are demonstrated to have a higher photosynthetic efficiency (Del Pozo et al., 2000). Although grasses were generally more flexible than legumes, when grown in mixture they had a more homeostatic shoot N:P (less reflective of substrate), which may provide evidence for facilitation (e.g. provision of N to grass) by the legume (Nyfeler et al., 2011; Wendling et al., 2017). This is however difficult to determine in this study without knowing the fate of applied fertilizers or biologically fixed N. Overall, with all other potentially limiting resources being equal (e.g. light, water), the ability of fast-growing, nutrient acquisitive species such as grasses to convert nutrients into biomass is likely to result in grass-dominated pasture systems (Haling et al., 2013).

Fertilisation in mixtures may lead to progressive N limitation in grasses despite their competitiveness

It is well-understood that grasses are primarily limited by N, and secondarily by P (Craine and Jackson, 2010), while legumes have higher requirements for P owing to its role in BNF (Heichel and Henjum, 1991). Therefore, P-only fertilisation in pasture mixtures may lead to progressive N limitation in grass (Agnusdei et al., 2010; Haynes, 1980), while N fertilisation can reduce the efficacy of symbiotic relationships (Mendoza et al., 2016a; Schomberg and Weaver, 1992). Growth limitation in our LNHP grass monoculture was relaxed by cultivation with legume, and shoot C:N ratios also decreased, indicating a reduced level of N limitation in mixtures. Further, the highest grass root C:N occurred in the LNLP grass monoculture, and

the lowest values in the LNLP mixture while, in the same LNLP mixture, legume root C:N concurrently increased (indicating limitation). It may be simple to suggest that cultivation with legume improved the ‘competitiveness’ of grasses, but in fact it may be that the two grass plants experienced less intraspecific competition for the soil available N than if the same amount of nutrient was available in monoculture with four grass plants given that legume plants have less demand for soil-derived N. Without the ability to trace the source and fate of nitrogen in this experiment, the conclusion that a facilitation effect occurred should be drawn with caution. There was no evidence of shoot or root P depletion in grass, and while P is generally demonstrated to favour legumes, N addition does not always favour grasses (Mendoza et al., 2016a). Here, N addition consistently favoured the grass, which increased the potential for P competition with the legume; this was further evidenced by decreased shoot C:P ratios in grasses grown in mixture. Increased grass competitiveness can occur if grass-legume mixtures are fertilized with both N and P (Mendoza et al., 2016b), possibly because N fertilization compromises BNF to a larger degree than P enhances it (Mendoza et al., 2016a). Further, P fertilisation may decrease colonisation of arbuscular mycorrhizal fungi (AMF) which can negatively affect nutrient acquisition for both species (Graham, 2000). Reduced plant-soil feedbacks in mixtures are more likely to negatively affect legumes because they rely more heavily on assimilating nutrients *via* symbiotic associations than nutrients supplied *via* mineral fertilisers (Nyfeler et al., 2009).

Legume root stoichiometry indicates potential for progressive nutrient limitation under low N and P conditions

Legume C:nutrient ratios in shoots and roots were not directly correlated with growth, suggest a more homeostatic nutrient acquisition strategy (Guo et al., 2017; Minden and Kleyer, 2014; Yu et al., 2011), and demonstrating the importance of considering interspecific

variation in nutrient use and allocation traits when assessing nutrient limitation. Legume shoot C:N ratios are often conserved despite fertilisation with N or P, or cultivation with grass (Bingcheng et al., 2018; Castellanos et al., 2018). However, in the present study, legume root stoichiometry did respond to N and P status, likely because legumes allocate more nutrients to roots to support nodule development, especially in nutrient-poor conditions (Vardien et al., 2016). In the control (LNLP) treatment, legume root C:N was significantly higher in mixture, while grass root C:N was lowest in the same treatment, indicating that some N depletion in the legume may have been occurring - despite their AGB remaining unaffected. Increases to legume root C:P ratios in N and P limited mixtures suggests that over time, progressive P depletion may occur in the legume. This could be due to grasses relying more strongly on the legume N source, or, because initial grass uptake of soil available N and associated growth can increase legumes' reliance on BNF over time, increasing their P requirement (Mendoza et al., 2016b).

Conclusions - Understanding plant-trait variation from an ecological stoichiometry perspective can inform pasture nutrient dynamics

Despite the usefulness of ecological stoichiometry in explaining nutrient limitation and competition potential in mixed pastures, it has been relatively underapplied in agricultural systems. Further, previous studies using ecological stoichiometry to demonstrate trait variation have not statistically tested their homeostatic coefficients (Mariotte et al., 2017; Riley et al., 2019; Yu et al., 2011), making comparisons among studies challenging. This study, while limited in its broader applicability to field systems given it was undertaken in controlled, glasshouse conditions allowed detection of differences between the tested species' growth and nutrient uptake traits and potential mechanisms of inter- and intraspecific nutrient competition. Considering plant trait variation in the context of stoichiometric

homeostasis is useful to tease out potential for nutrient competition in mixed communities containing species with significantly different functional traits. These results must be interpreted cautiously to infer species-level dynamics; follow-up studies should include testing stoichiometric theory in agricultural field-based trials, using multiple planting densities and nutrient applications and, to deepen mechanistic understanding, include isotopically labelled fertilizers.

Declarations

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No custom code was produced for this manuscript.

Authors' contributions: KB designed and managed the experiment, performed statistical analyses, and wrote the manuscript. SW consulted on the experimental design and directed the development of the manuscript. SP and EP supervised the progress of the experimental design and statistical analyses and directed the development of the manuscript. BB consulted on experimental design, managed the experiment and directed the development of the manuscript. CB produced the experimental design, performed the linear mixed model analyses and consulted on the manuscript.

The authors wish to recognize that this study was conducted at the University of Adelaide on the traditional lands of the Kaurna people.

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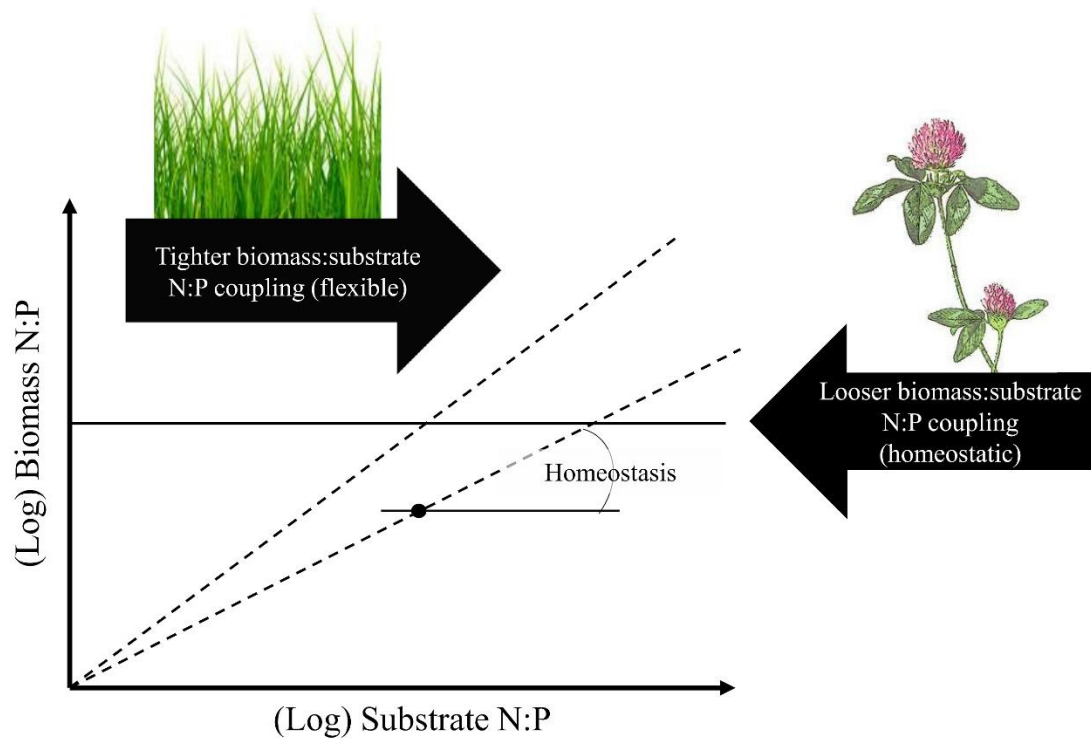


Figure 1: Stoichiometric theory in the experimental context. The coupling of N:P ratios between tissues and substrate ranges from no relationship (————) to flexibility (1:1 relationship; - - - - -). The degree of homeostatic regulation is often determined using the inverse of the slope ($1/\text{slope}$) and is reported relatively, with larger numbers indicating stricter homeostasis (Sterner, 2002). Grasses are likely more homeostatically flexible species, and legumes more homeostatically strict.

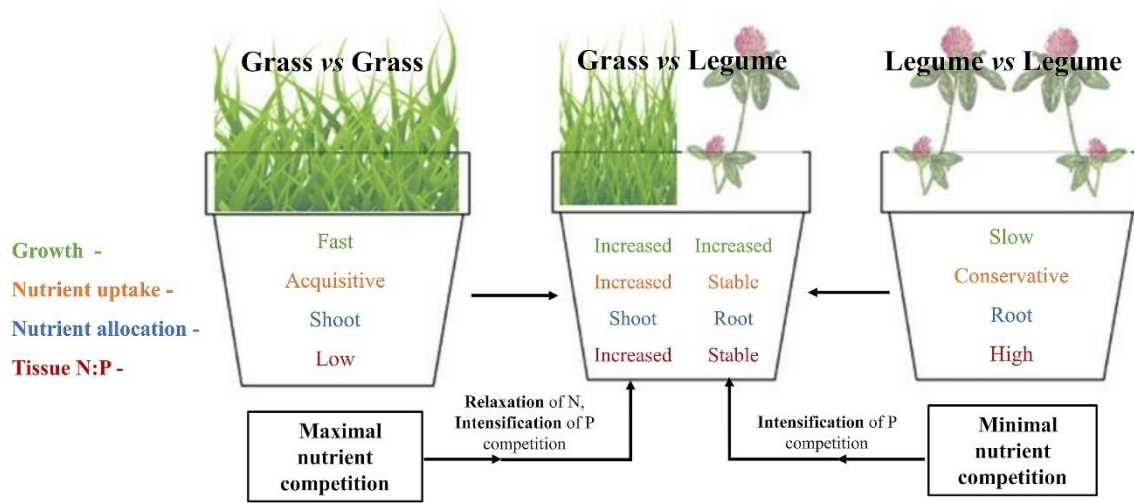


Figure 2: Experimental design indicating **a)** conceptual plant traits (left and right pots) and hypothesised trait response changes between monoculture and mixture (middle pot), **b)** variations to nutrient competition between cultivation types.

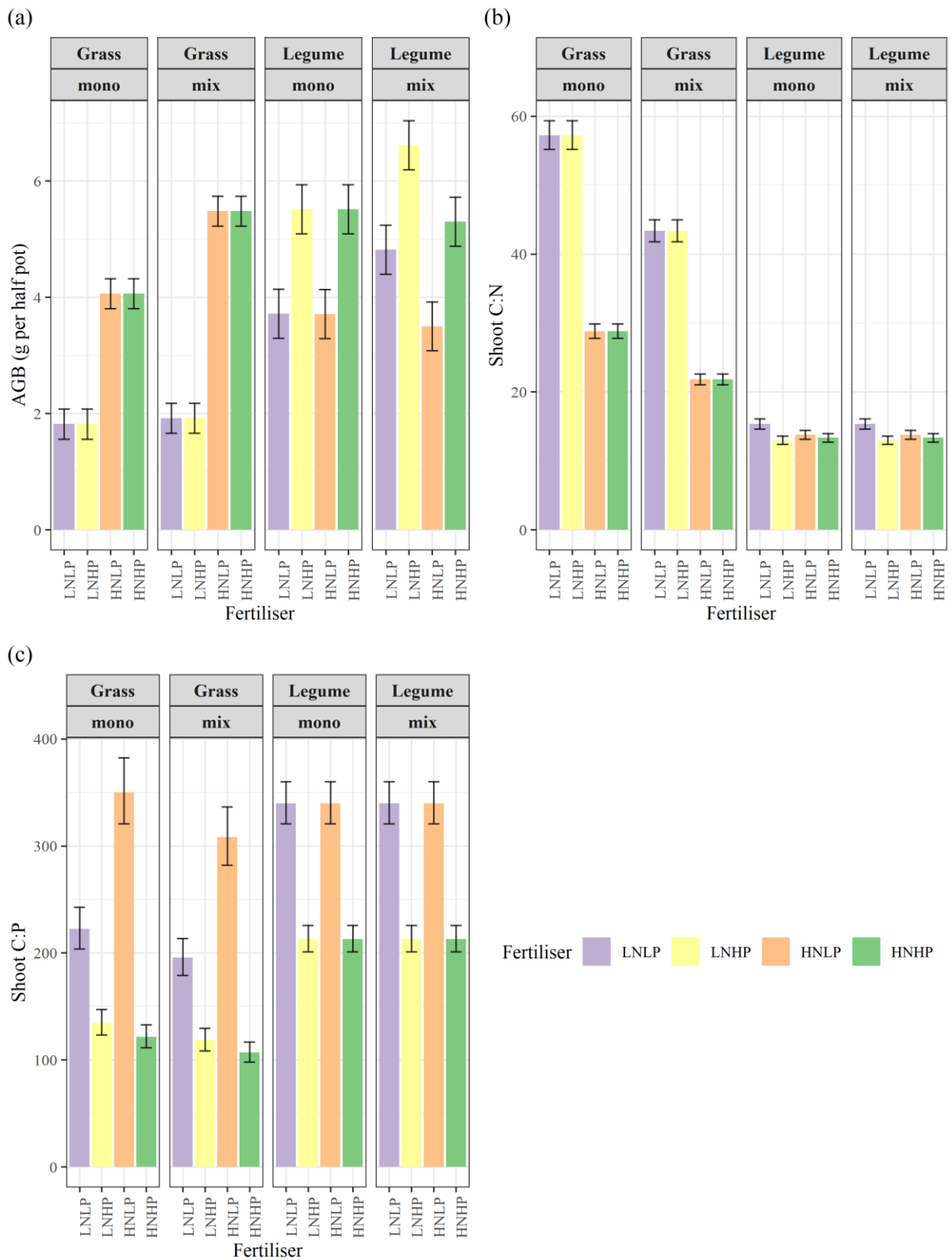


Figure 3: Model estimated marginal means (\pm half-Least Square Differences [5%]) for the aboveground traits a) Above ground biomass (AGB, g per half-pot) b) shoot C:N ratio c) shoot C:P ratio for grass and

legume in monocultures (mono) and mixtures (mix). Non-overlapping error bars indicate significant differences at $\alpha = 0.05$.

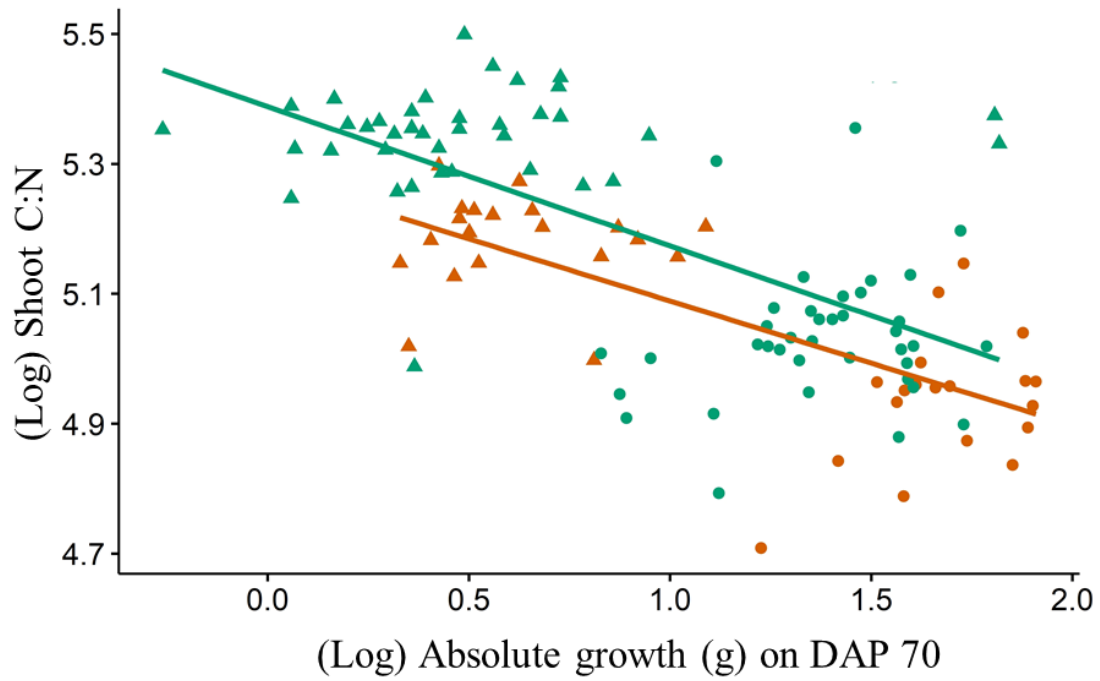


Figure 4: The relationships between shoot C:N and aboveground biomass (g) in grass. Monoculture (green) and mixture (orange). High N treatments = circles, Low N treatments = triangles.

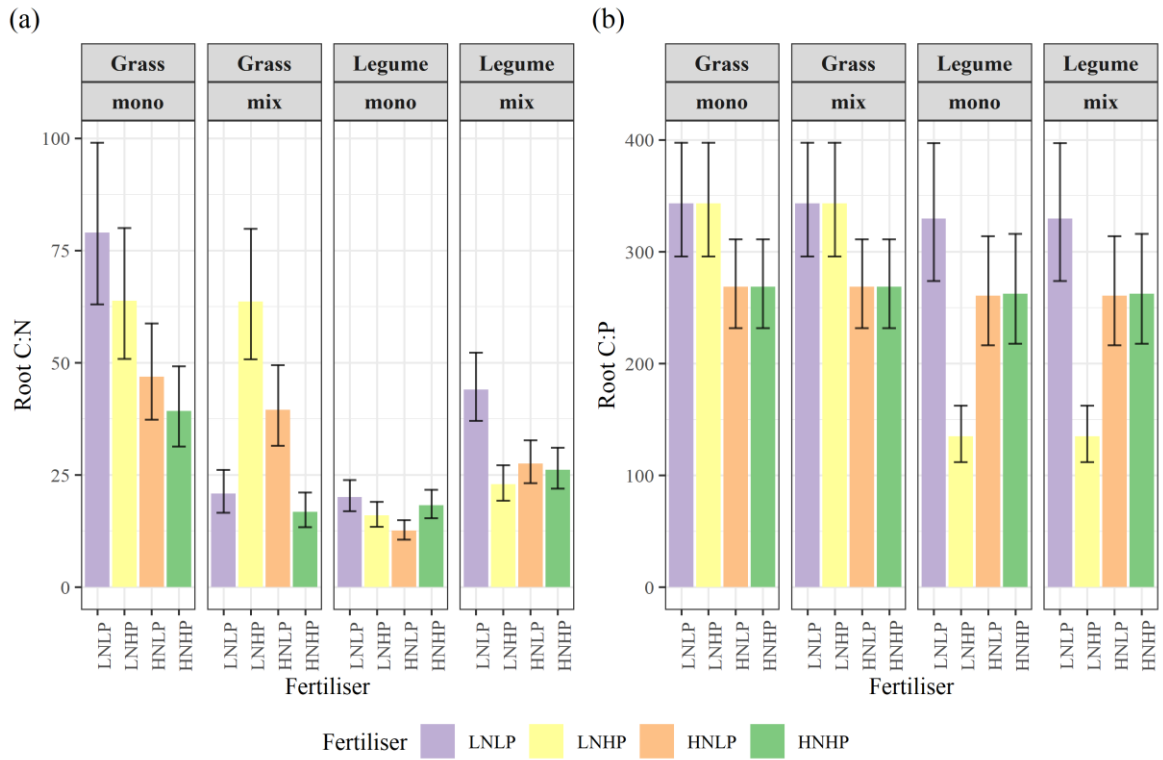


Figure 5: Model estimated marginal means (\pm half-Least Square Differences [5%]) for the belowground traits a) root C:N ratio c) root C:P ratio for grass and legume in monocultures (mono) and mixtures (mix). Non-overlapping error bars indicate significant differences at $\alpha = 0.05$.

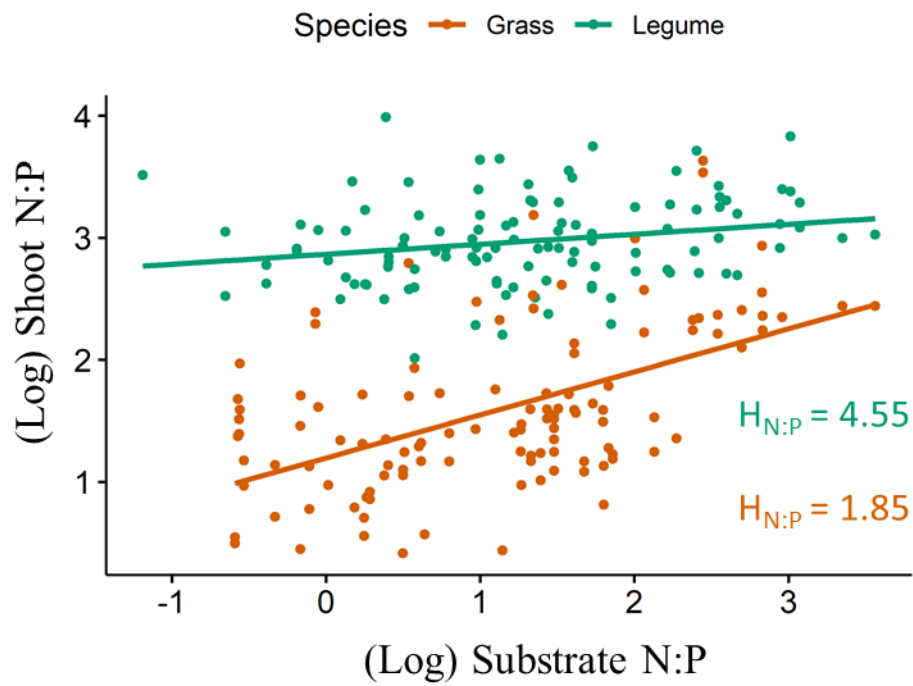
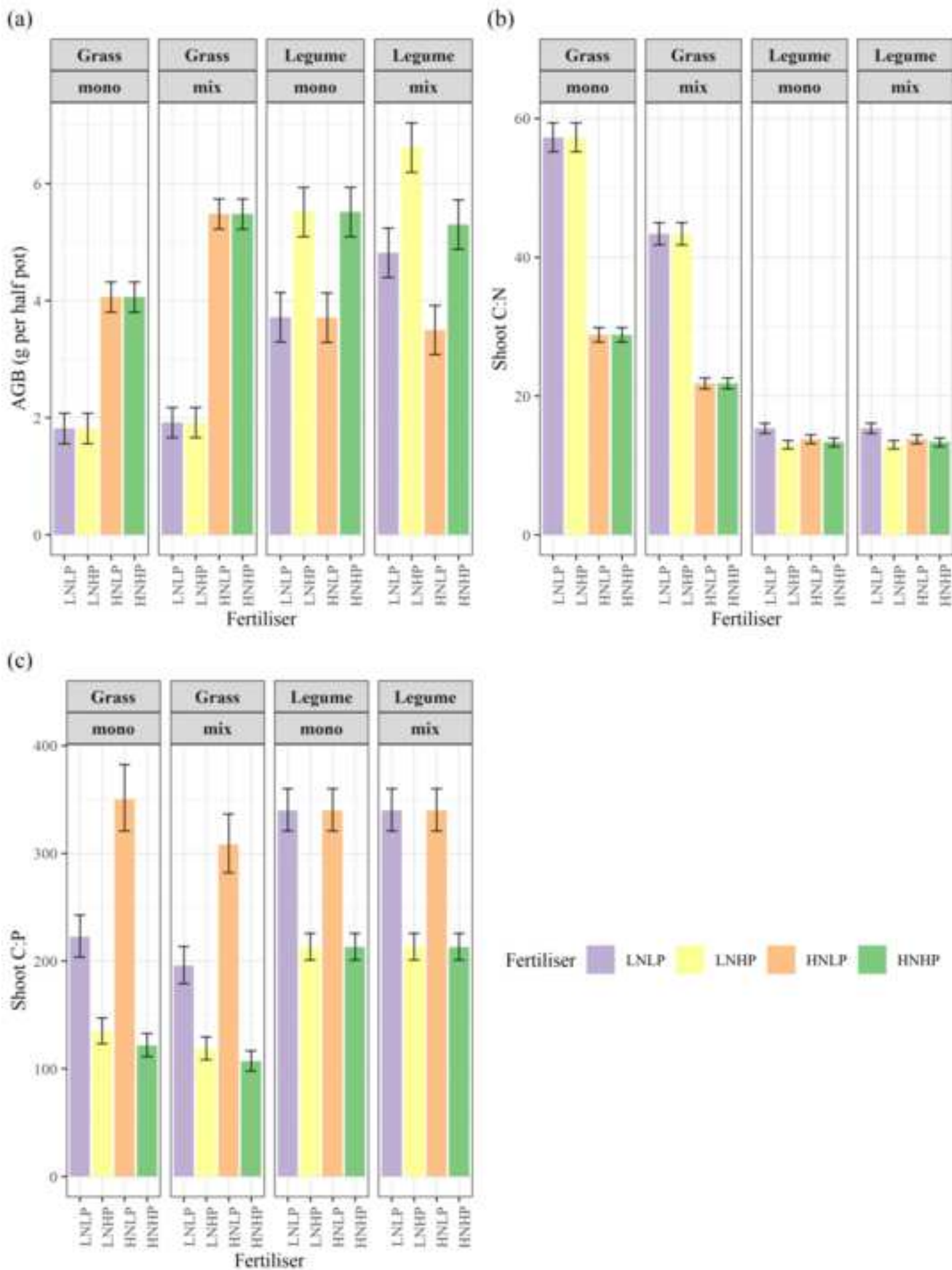
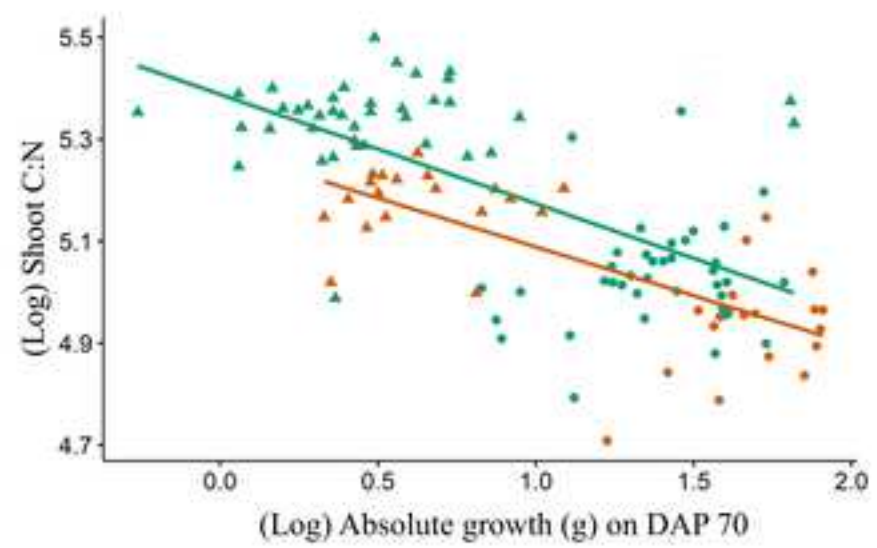
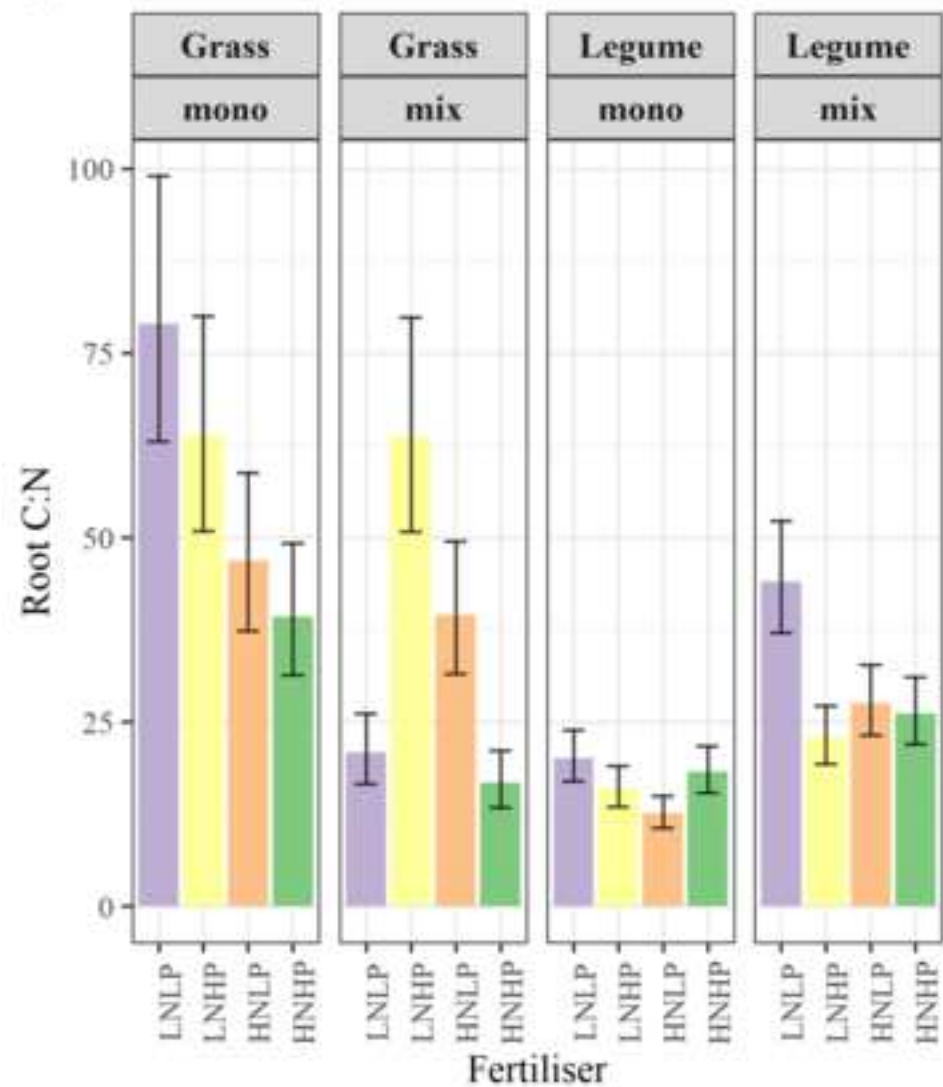


Figure 6: Shoot N:P homeostasis (mass basis) between species. Slope of the relationship for legume is closer to zero (with a higher $H_{N:P}$ coefficient), indicating tissue N:P is more loosely coupled with substrate N:P (more homeostatic). Lower $H_{N:P}$ coefficient of grass indicates that N:P homeostasis is more flexible.



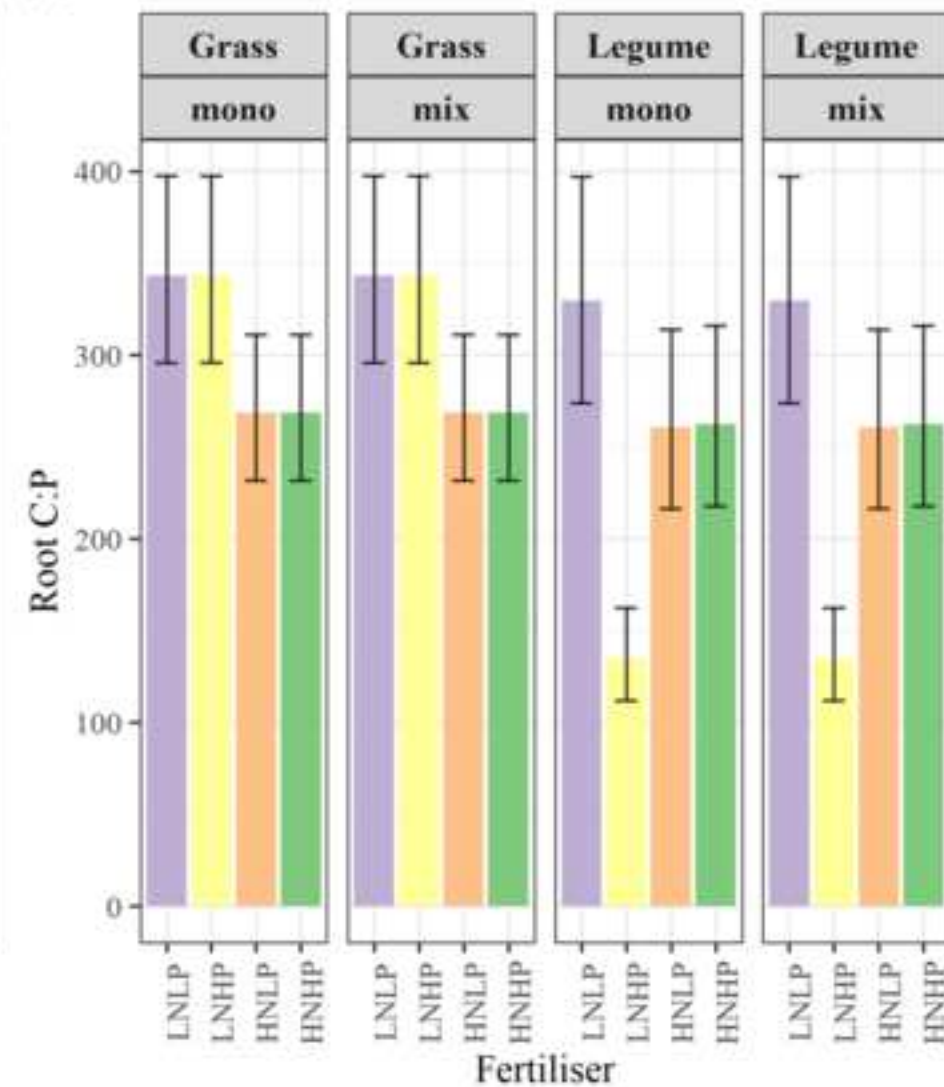


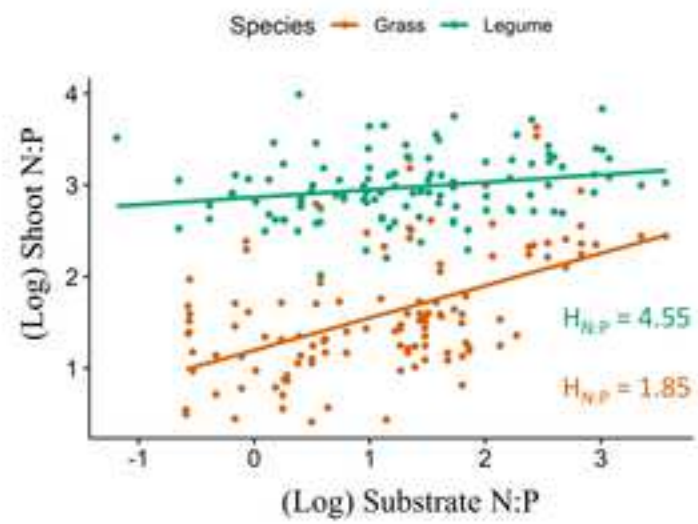
(a)



Fertiliser LNL LNHP HNLP HNHP

(b)

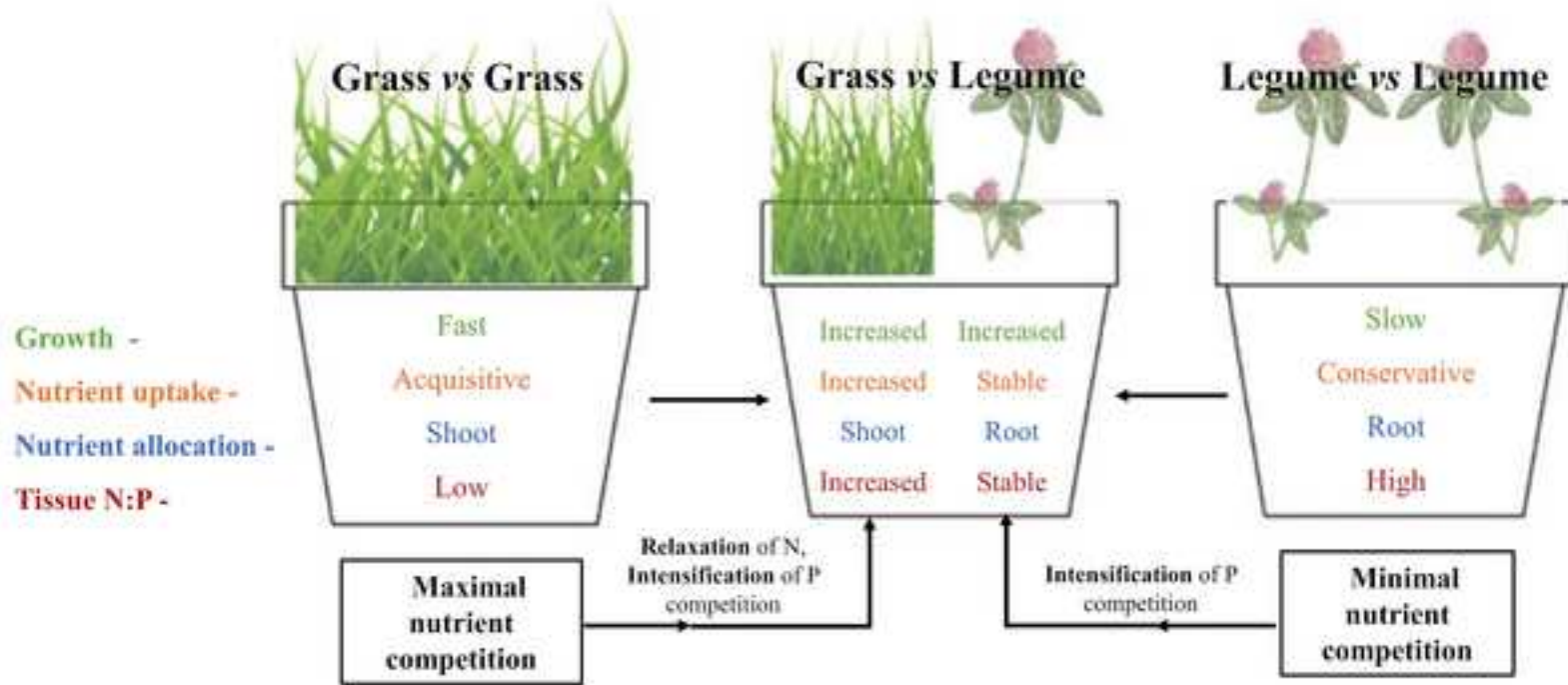


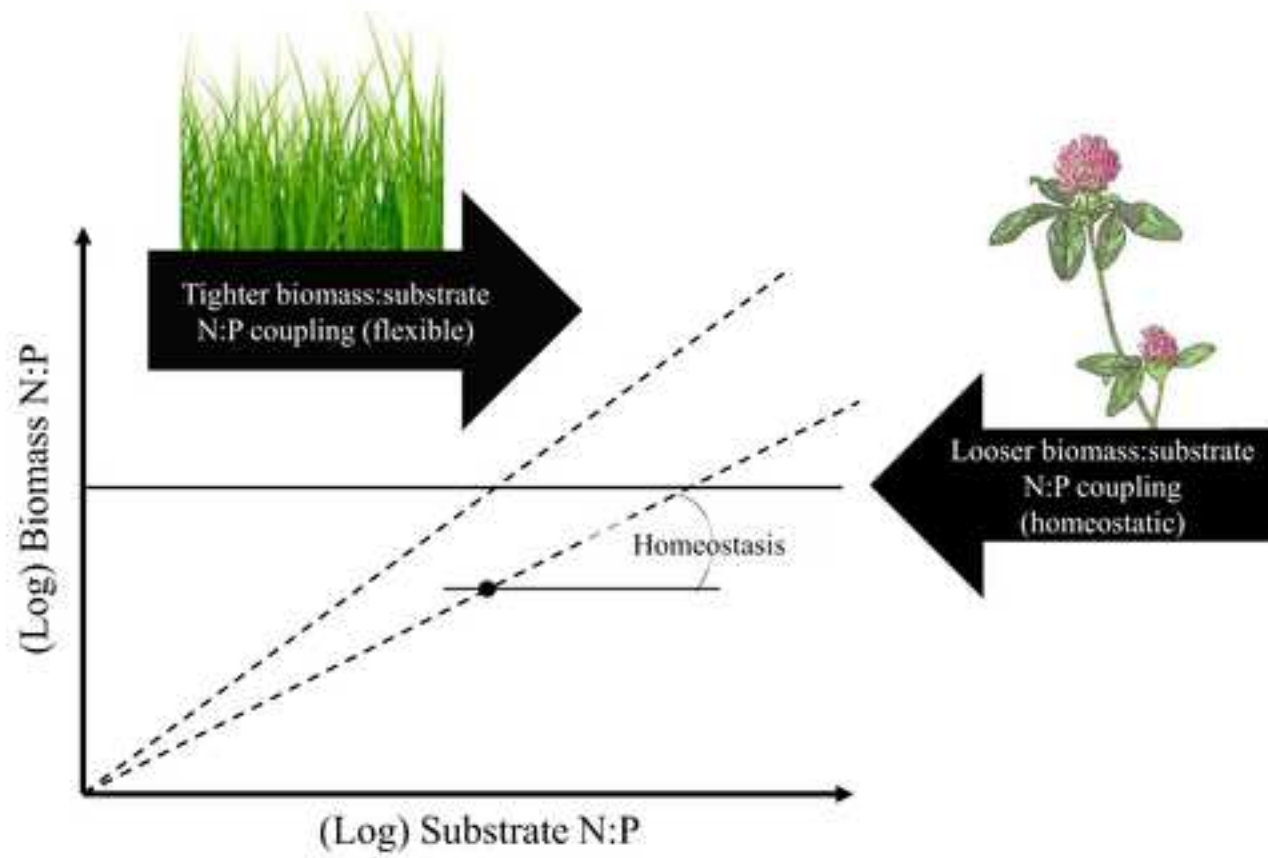


Positions, Halves

24W 24E 22W 22E 20W 20E 18W 18E 16W 16E 14W 14E 12W 12E 10W 10E 8W 8E 6W 6E 4W 4E 2W 2E

		Gra	Gra	Leg	Leg	Leg	Gra	Gra	Gra	Gra	Leg	Leg	Leg	Leg	Leg	Gra	Gra	Leg	Leg	Gra	Gra	Gra	Leg
11		HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP
		Gra	Gra	Gra	Leg	Leg	Leg	Leg	Leg	Gra	Gra	Leg	Gra	Leg	Leg	Gra	Gra	Leg	Leg	Gra	Gra	Leg	Gra
10		LNLP	LNLP	LNLP	LNLP	LNLP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP
		Leg	Leg	Gra	Gra	Gra	Leg	Leg	Leg	Leg	Gra	Gra	Gra	Gra	Gra	Gra	Gra	Leg	Leg	Gra	Leg	Gra	Gra
9		HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP
		Leg	Gra	Gra	Gra	Leg	Leg	Leg	Leg	Gra	Leg	Gra	Gra	Leg	Leg	Gra	Gra	Leg	Gra	Gra	Gra	Leg	Leg
8		HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP	HNLP
		Gra	Leg	Leg	Leg	Gra	Gra	Leg	Leg	Gra	Gra	Leg	Gra	Leg	Leg	Gra	Leg	Leg	Leg	Gra	Gra	Leg	Gra
7		LNLP	LNLP	LNLP	LNLP	LNLP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP
		Leg	Gra	Leg	Leg	Gra	Gra	Gra	Gra	Gra	Leg	Leg	Leg	Gra	Leg	Leg	Gra	Leg	Leg	Leg	Leg	Gra	Gra
6		HNLP	HNLP	HNLP	HNLP	HNLP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP
		Gra	Gra	Leg	Leg	Leg	Gra	Gra	Gra	Gra	Leg	Leg	Leg	Gra	Gra	Leg	Leg	Leg	Leg	Gra	Leg	Gra	Gra
5		LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP
		Gra	Gra	Gra	Leg	Leg	Leg	Leg	Gra	Leg	Leg	Gra	Gra	Leg	Leg	Gra	Gra	Gra	Gra	Leg	Gra	Leg	Leg
4		HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP
		Leg	Leg	Gra	Gra	Gra	Leg	Leg	Leg	Leg	Gra	Gra	Gra	Gra	Gra	Gra	Gra	Gra	Gra	Leg	Leg	Leg	Gra
3		LNLP	LNLP	LNLP	LNLP	LNLP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP	LNHP
		Leg	Leg	Gra	Gra	Leg	Gra	Gra	Gra	Gra	Leg	Leg	Leg	Gra	Gra	Gra	Gra	Gra	Gra	Leg	Leg	Gra	Leg
2		HNLP	HNLP	HNLP	HNLP	HNLP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP	HNHP







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Supplementary Material

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