

Contrasting responses of crop legumes and cereals to nitrogen availability

Mark A. Adams¹, Thomas N. Buckley², William T. Salter³, Nina Buchmann⁴, Carola H. Blessing³ and Tarryn L. Turnbull³

¹Department of Chemistry and Biotechnology, Faculty of Science, Engineering and Technology, Swinburne University, PO Box 218, Hawthorn, Vic 3122, Australia; ²Department of Plant Sciences, University of California, Davis, One Shields Ave, Davis, CA 95616, USA; ³School of Life and Environmental Sciences, Faculty of Science, The University of Sydney, 380 Werombi Rd, Camden, NSW 2570, Australia; ⁴ETH Zurich, Universitätsstrasse 2, 8092, Zürich, Switzerland

Author for correspondence:
Mark A. Adams
Tel: +614 2101 1169
Email: maadams@swin.edu.au

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Summary

- In nonagricultural systems, the relationship between intrinsic water-use efficiency (WUE_i) and leaf nitrogen (N_{area}) is known to be stronger for legumes than for nonlegumes. We tested whether these relationships are retained for major agricultural legumes and nonlegumes.
- We compared the response to N nutrition of WUE_i (and its component parts, photosynthesis (A_{sat}) and stomatal conductance (g_s)) for legumes *Cicer arietinum*, *Glycine max*, *Lupinus alba* and *Vicia faba*, nonlegume dicots *Brassica napus* and *Helianthus annuus*, and nonlegume cereals *Hordeum vulgare* and *Triticum aestivum*.
- Surprisingly, and in contrast to studied cereals and nonlegume dicots, N_{area} was positively related to photosynthesis in the legumes, explaining nearly half of the variance in A_{sat} . WUE_i was tightly coupled to N_{area} for agricultural legumes and nonlegume dicots, but not for cereal crops. Our analysis suggests that breeding efforts to reduce g_s in legumes could increase WUE_i by 120–218% while maintaining A_{sat} at nonlegume values.
- Physiologically informed breeding of legumes can enhance sustainable agriculture by reducing requirements for water and N.

Introduction

Availability of water and nitrogen (N) are the greatest constraints to growth and primary productivity of agricultural crops across the globe (Mueller *et al.*, 2012). Each year, growing cereal grains to meet (current) global food demand requires *c.* 2500 Gm³ of water (equating to 27% of the global annual water consumption; Hoekstra & Mekonnen, 2012), and over 100 Mt of N fertilizer (Foyer *et al.*, 2016). Consequently, considerable effort is being directed towards breeding crops (Condon *et al.*, 2004) and managing agricultural systems for both water- and N-use efficiency (Quemada & Gabriel, 2016).

In natural ecosystems, most legumes have a reduced requirement for N fertilizer through their symbiotic relationship with N-fixing diazotrophs, and their resulting increased leaf N content helps to directly mitigate leaf water losses; for a given rate of photosynthesis, stomatal opening can be reduced (Adams *et al.*, 2016). In agriculture, and especially in cropping systems, grain legumes could be playing a greater role in meeting global aims of increased, yet sustainable, production of nutritious food. Whilst decades of research into the physiology of cereal grains has ensured the development of agronomic practices that increase yield, research into the physiology of grain legumes is comparatively scant. A consequence is that global increases in grain legume yields are currently met almost exclusively through

increased planting area (Foyer *et al.*, 2016). Improved understanding of physiological differences between grain legumes and cereal grains and other nonleguminous crops is urgently required to increase the effectiveness of legume breeding programs focused on increasing yield while conserving water and N resources.

Leaf N content (expressed per unit leaf area, N_{area}) has often been described as positively related to primary growth and productivity, as photosynthetic enzymes and pigments can make up *c.* 70% of leaf N (Rubisco and Chl; Field & Mooney, 1986), and increased leaf N usually supports increased photosynthetic capacity (Evans, 1989). Concomitantly, increased photosynthesis consumes intercellular CO₂ at a greater rate and generates a steep CO₂ diffusional gradient between the intercellular air space and the atmosphere, which in turn allows for reduced stomatal aperture (Farquhar *et al.*, 2002) and increased intrinsic water-use efficiency (WUE_i) (Stitt & Schulze, 1994). Whilst this scenario best describes sunlit conditions where carboxylation is limiting, Kromdijk *et al.* (2016) recently suggested increased WUE_i in partly shaded lower canopy or sunfleck conditions could improve if breeding were to focus on traits associated with rapid recovery from photoinhibition, processes also known to require investment of N (Raven, 2011). The oft-cited relationship between N_{area} and photosynthesis (e.g. Wright *et al.*, 2004) is not always supported by the literature, however. N is required for many metabolic processes (e.g. respiration, Reich *et al.*, 2008), as well as for structure and defense, and these

uses introduce considerable variation in the response of photosynthesis to increased N_{area} (Meziane & Shipley, 2001; Dong *et al.*, 2017; Onada *et al.*, 2017).

We recently evaluated the literature for legumes and nonlegumes from native ecosystems and showed that N_{area} is only a reasonable predictor of area-based rates of photosynthesis (A_{sat}) for the latter. N_{area} and A_{sat} were unrelated for these mostly woody plants. Curiously, N_{area} explained more of the variation in WUE_i in legumes than in nonlegumes (Adams *et al.*, 2016). It is reasonable to hypothesise that physiological responses to N of legume and nonlegume crop species should also differ. However, very few studies have directly compared these functional groups.

The limited literature available (including measures of N_{area} , A_{sat} and stomatal conductance, g_s) has suggested the following responses to added N by cropping species: A_{sat} , g_s and WUE_i were unaffected by added N for *Glycine max* (Moreira *et al.*, 2015); A_{sat} increased and g_s decreased for *Cicer arietinum* (Tak *et al.*, 2010); A_{sat} increased and g_s was invariant for *Phaseolus vulgaris* (Jifon & Wolfe, 2002); and water loss increased for *Phaseolus vulgaris* (Shimshi, 1970). Similarly contrasting findings characterize the literature on the physiological responses of cereal grains and dicot nonlegumes to N nutrition (e.g. Lopes *et al.*, 2004; Lopes & Araus, 2006). For example, increased N supply to wheat (*Triticum aestivum*) and durum wheat (*Triticum turgidum* L. ssp. *durum*) can increase A_{sat} (Shangguan *et al.*, 2000; Del Pozo *et al.*, 2007; Cabrera-Bosquet *et al.*, 2009), g_s (Shangguan *et al.*, 2000), WUE_i (Cabrera-Bosquet *et al.*, 2007) and mesophyll conductance (Barbour & Kaiser, 2016). However, opposite effects have also been observed. For example, the response of cereal grains and nonlegume dicots to N nutrition includes reduced A_{sat} (Cabrera-Bosquet *et al.*, 2007) and g_s (Cabrera-Bosquet *et al.*, 2007, 2009). In other studies there has been no response of A_{sat} (van den Boogaard *et al.*, 1995) or g_s (van den Boogaard *et al.*, 1995; Del Pozo *et al.*, 2007) to enhanced N supply. The literature is similarly mixed for other cereals; photosynthesis increases with N supply in rice (*Oryza sativa*) (Guo *et al.*, 2011; Li *et al.*, 2013), whereas g_s can be invariant (Guo *et al.*, 2011; Li *et al.*, 2013) or increase (Li *et al.*, 2013). For the dicot nonlegumes, for example *Helianthus annuus*, A_{sat} (Fredeen *et al.*, 1991) can increase or remain invariant (Zeng *et al.*, 2014) with increased N nutrition, whilst g_s appears unchanged by N nutrition (Fredeen *et al.*, 1991; Zeng *et al.*, 2014) such that WUE_i usually increases (Fredeen *et al.*, 1991; Cechin & Fumis, 2004). Lone & Khan (2007) noted that A_{sat} , but neither g_s nor WUE_i , followed N supply in *Brassica juncea*.

Hence, whilst photosynthetic responses to exogenous N of nonlegumes, and, to a lesser extent, of legumes, have received some attention, a robust reconciliation of differences among the two functional groups has not been achieved. Large variation among studies in growth mediums, light environments (during both growth and measurement), growth stage at which measurements were made, and N application rate contributes to large variability in results. Almost every possible permutation of responses in gas-exchange parameters has been recorded. No one study has, as far as we can tell, directly compared (grown and measured under the same conditions) basic physiological

responses to N such as N_{area} , A_{sat} , g_s and thus WUE_i in leguminous and nonleguminous agricultural crops.

We measured the response of leaf N, A_{sat} , g_s and WUE_i to N nutrition in eight major agricultural crops – four legumes and four nonlegumes – grown concurrently under natural insolation. Measurements were made pre-anthesis to represent the period of greatest water use – and which best reflects water available for plants during periods critical to crop yield (anthesis and grain fill; Vadez *et al.*, 2014 and references therein). We sought to test whether the photosynthetic physiology of grain legumes would differ in response to N nutrition, as compared with monocot cereals or dicot nonlegumes. Specifically, we hypothesized that the cereals and dicot nonlegumes, having been bred for many decades for increased yields, would retain a positive relationship between A_{sat} and N_{area} , whereas grain legumes would resemble native legumes in using N_{area} to increase WUE_i more so than A_{sat} .

Materials and Methods

Plant material and experimental design

Experiments were conducted outdoors at the University of Sydney's Centre for Carbon Water and Food, NSW, Australia (34°2'4''S, 34°39'29''E). We studied major agricultural species from a variety of natural origins, including the legumes *Cicer arietinum* (chickpea, Middle East) and *Vicia faba* (broadbean/fababean, Middle East), *Glycine max* (soybean, China) and *Lupinus alba* (lupin, western Asia/south east Europe) and dicotyledonous agricultural nonlegumes (nonlegume dicots) *Brassica napus* (canola, east Asia/southwest Europe) and *Helianthus annuus* (sunflower, North America), and nonlegume monocots (cereals) *Hordeum vulgare* (barley, western Asia) and *Triticum aestivum* (wheat, western Asia). Whilst there are now many genotypes of each study species, as well as a variety of common forms (e.g. *Vicia faba*), we used one genotype for each species. Similarly, whilst seed size contributes to the supply of N for early growth, and clearly varies among (e.g. *Vicia faba* vs *Brassica napus*) and within the study species (e.g. *Cicer arietinum*; Foyer *et al.*, 2016) we did not directly address this potential source of variation. Addressing both within-species genetic variability and seed-size variation, in addition to the among species/functional group variation studied here, would require literally thousands of pots/growth chambers.

Seeds for all species were sown into 8 l pots filled with a mixture of peat, sand and perlite (20 : 30 : 50 v/v). Pots (eight replicates for each species and treatment) were randomly assigned to a nutrient regime – full-strength Hoagland's solution (with 8 mM N applied as NH_4NO_3) or a Hoagland's solution modified only for [N] (half-strength [N] (4 mM N), quarter-strength [N] (2 mM N), one-eighth-strength [N] (1 mM N) and no additional N (0 mM N)) – and arranged in a randomized block design, with species randomly allocated within blocks for nutrient treatment. Pots were watered to field capacity twice daily, and with nutrient solution every second day for the duration of the experiment. Average day temperature was 30°C – close to the upper productive limit for the study species (Luo, 2011; Bishop *et al.*, 2016) –

relative humidity was 74%, and plants received in excess of $2000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ photon flux density during daylight hours via natural sunlight.

We measured gas exchange on five to seven plants (one per pot) for each species in each treatment (Supporting Information Table S1) between 23 and 30 d after germination when all plants were still pre-anthesis. Following measurement, the leaf used for photosynthesis was used to calculate specific leaf area (SLA, $\text{m}^2 \text{kg}^{-1}$) before being ground to a fine powder for total N content (%N) and stable carbon ($\delta^{13}\text{C}$) and N ($\delta^{15}\text{N}$) isotope composition on an isotope ratio mass spectrometer (Delta V, Thermo Finnigan, Bremen, Germany). Four to five of the remaining plants for each species in each treatment (Table S1) were then harvested and separated into leaves, stems and roots before drying at 65°C for 72 h and weighing for dry biomass, and analyzing separate plant fractions for N content.

Gas exchange

All measurements were made outdoors in saturating light conditions between 10:00 and 13:00 h on the youngest fully expanded leaves with two infrared gas analyzers (Li-Cor 6400; Li-Cor, Lincoln, NE, USA) each fitted with a 6 cm^2 cuvette and a red/blue LED light source. During measurements, the cuvette was set to a CO_2 partial pressure of 400 ppm and a photosynthetic photon flux density of $2000 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, and leaf temperature was controlled to $24.5 \pm 0.1^\circ\text{C}$, equating to an average vapor pressure deficit of $1.30 \pm 0.02 \text{ kPa}$.

To simulate the gains in WUE_i that would result from breeding for reduced g_s in legumes, we interpolated observed relationships between A_{sat} and c_i (intercellular CO_2 concentration) to find the values of c_i at which A_{sat} would be reduced by 23.32% – the percentage by which A_{sat} was lower in nonlegumes than in legumes under zero added N in the experiment described earlier. We generated composite response curves by fitting quadratic regressions to four to five A_{sat} vs c_i response curves per species, averaging these curves' predictions for each species at each of a range of c_i values between 10 and 390 ppm, and fitting quadratic regressions to the resulting average A_{sat} vs c_i relationships. We then applied mean *in situ* values of c_i measured under saturating light for six to seven leaves of each species to these composite response curves to estimate an initial A_{sat} for each legume species. We reduced these initial values of legume A_{sat} by 23.32% to represent the effect of a hypothetical reduction of g_s in legumes, and calculated the corresponding value of c_i by applying the reduced A_{sat} value to the composite A_{sat} vs c_i relationship. Finally, for both initial and reduced A_{sat} conditions, we estimated g_s as $1.6/((c_a - c_i)/A_{\text{sat}} - 1/g_{\text{bc}})$ (where $c_a = 400 \text{ ppm}$ is the ambient CO_2 concentration, and $g_{\text{bc}} = 2.92 \text{ mol m}^{-2} \text{s}^{-1}$ is the boundary layer conductance to CO_2 , approximately the mean value in the Li-6400 chambers used in the experiments), and then calculated WUE_i as A_{sat}/g_s .

Data analysis

Shapiro–Wilk tests showed that the data for g_s , WUE_i and N_{mass} were significantly nonnormal (skewed to the right); \log_{10}

transformations improved normality distributions of these data. We used multivariate analyses (linear mixed models, maximum likelihood) to examine the influence of crop type (legume, non-legume dicot, cereal) and N nutrition (nutrient regime: 0, 1, 2, 4 or $8 \text{ mol m}^{-3} [\text{N}]$) as fixed factors on leaf physiology, chemistry and plant pools of N. Species were assigned as random factors for all analyses to counter nonindependence. Additionally, we assessed the possibility that accounting for possible species \times N interaction would provide additional insight, by repeating each analysis with a species \times N interaction term as a random variate. However, for all dependent variables (except $\delta^{15}\text{N}$), the resulting models were less parsimonious (higher Akaike information criterion (AIC)) than when species was used alone as the random factor, suggesting that accounting for the interaction did not improve our analysis (see also Table S2). Differences were identified as statistically significant for $P < 0.05$. We also used Pearson correlations to examine bivariate relationships among measures of water-use efficiency and leaf N for each crop type. Lastly we used ANOVA to identify differences in capacity to fix N among legume species with varying concentrations of added N. All analyses were performed with SPSS v.19 (IBM Australia Ltd, St Leonards, NSW, Australia).

Results

Nitrogen nutrition influenced the capacity of all crops to accumulate biomass (Table S3) and N (Fig. 1a–c). All crops accumulated the most N when the supply of exogenous N was greatest; we observed fourfold greater total plant N in the $8 \text{ mol m}^{-3} [\text{N}]$ treatment than in the $0 \text{ mol m}^{-3} [\text{N}]$ treatment for legume crops. The respective increase in cereals was 50-fold, and in the non-legume dicots was 60-fold ($P < 0.001$, Fig. 1a). Predictably, we observed significant interaction between crop type and N nutrition in the capacity to amass plant N in leaves ($P < 0.001$, Fig. 1b) and roots ($P < 0.01$, Fig. 1c), owing to the capacity for legume crops to fix N when no exogenous N was supplied (as indicated by significant differences in leaf $\delta^{15}\text{N}$ among crop types; $P < 0.05$) – a capacity that is diminished with increasing rate of $[\text{N}]$ addition above 2 mM (Fig. 1d).

In the absence of exogenous (applied) N, N_{area} and N_{mass} of legumes were also significantly greater than those of their non-legume counterparts (indicated by significant $P < 0.05$ interaction, Fig. 2a,b). Legume crops contained $1.7 \text{ g m}^{-2} \text{N}$ per unit area (N_{area}), 40% more than cereal and nonlegume dicot crops (both $1.2 \text{ g m}^{-2} \text{N}$) in the $0 \text{ mol m}^{-3} [\text{N}]$ treatment (Fig. 2a). Expressing data on a mass basis further amplified the insensitivity of legume crops to artificially enhanced N supply: legume crops contained $34.6 \text{ mg g}^{-1} \text{N}$ per unit leaf mass (N_{mass}), twice that of cereals (17.4 mg g^{-1}), and 50% more than nonlegume dicot crops (23.7 mg g^{-1}) in the $0 \text{ mol m}^{-3} [\text{N}]$ treatment (Fig. 2b).

All crops up-regulated photosynthesis with added N ($P < 0.001$). However, in the absence of added N, legume crops displayed greater photosynthetic rates than nonlegumes (indicated by significant $P < 0.05$ interaction, Fig. 3a). Similarly, all crops increased stomatal conductance to water vapor with added N (Fig. 3b, $P < 0.001$), but differences among crop types

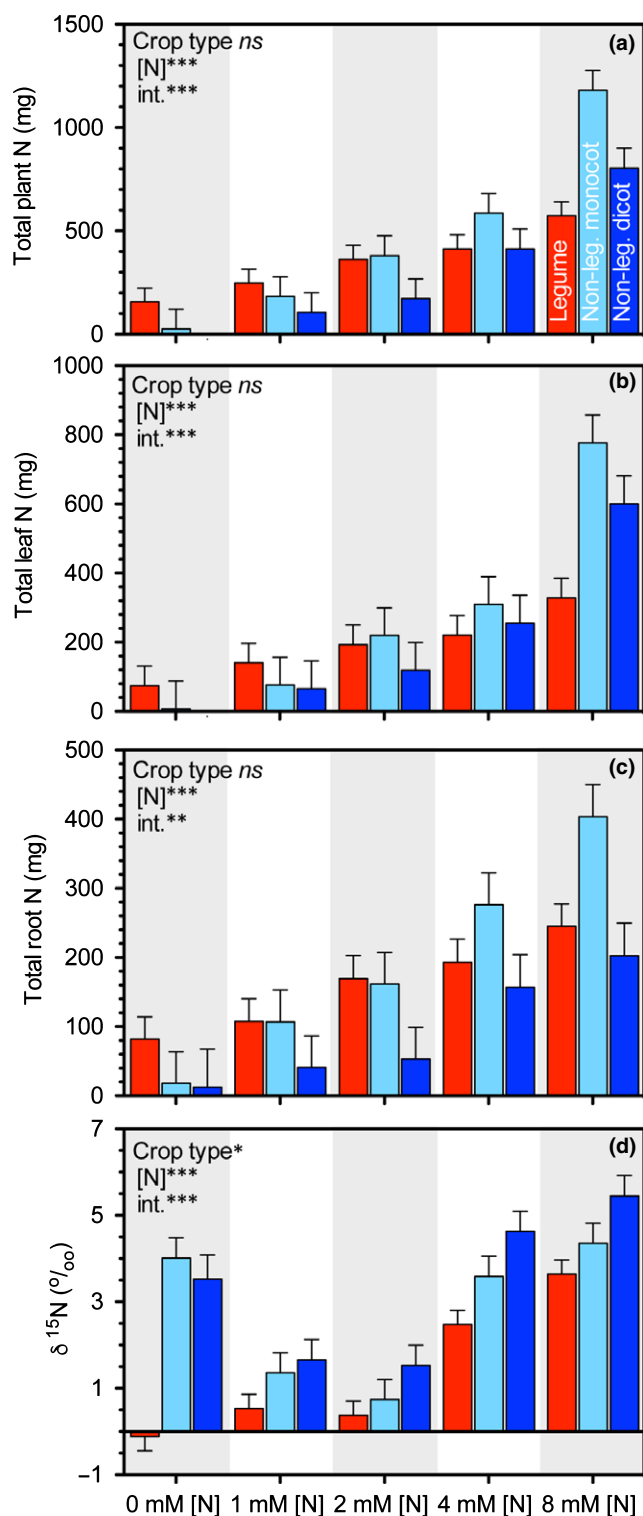


Fig. 1 Accumulation and source of nitrogen (N) in legume (red), nonlegume dicot (royal blue) and cereal (nonlegume monocot, light blue) agricultural crops from our controlled environment comparison. (a) Total plant N (mg); (b) N contained in leaves (mg); (c) N contained in roots (mg); (d) N isotope discrimination ($\delta^{15}\text{N}$, ‰) of leaf tissue. Data shown are estimated marginal means and SE for four to five replicates per species per treatment (see Supporting Information Table S1) from linear mixed models with crop type, N-addition rate ([N]) and their interaction as fixed effects and species as a random factor. ns, not significant ($\alpha = 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

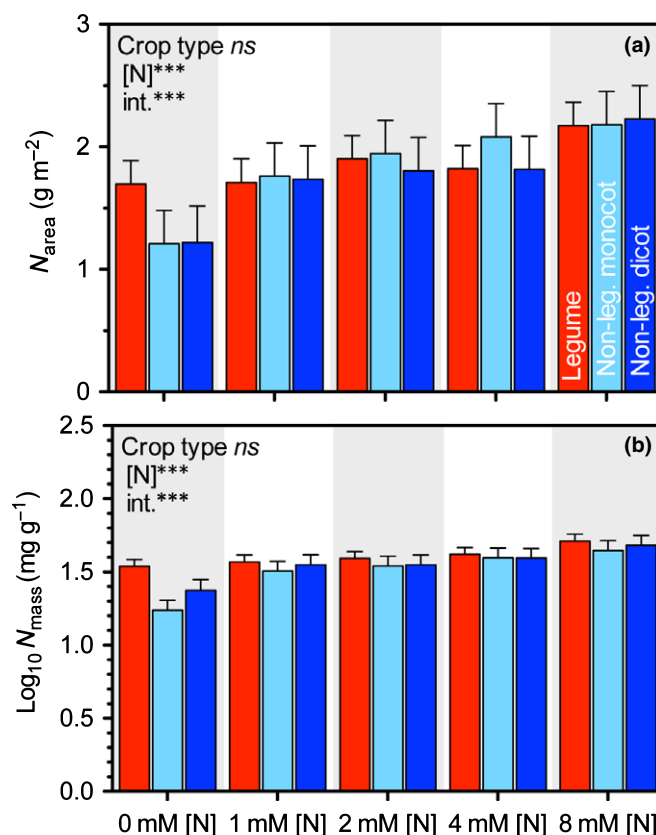


Fig. 2 Measures of leaf nitrogen (N) for legume (red), nonlegume dicot (royal blue) and cereal (nonlegume monocot, light blue) agricultural crops from our controlled-environment comparison. (a) Leaf N per unit area (N_{area} , g m^{-2}); (b) leaf nitrogen per unit mass (N_{mass} , mg g^{-1}). N_{mass} data were \log_{10} -transformed before analyses to improve normality distributions. Data shown are estimated marginal means and SE for between five and seven replicates per species per treatment (see Supporting Information Table S1) from linear mixed models, with crop type, nitrogen addition rate ([N]) and their interaction as fixed effects and species as a random factor. ns, not significant ($\alpha = 0.05$); ***, $P < 0.001$.

(cereal < legume < nonlegume dicot, $P < 0.05$) were independent of N treatments. Curiously WUE_i did not improve with added N; irrespective of treatment, the WUE_i of cereals = legumes < nonlegume dicots ($P < 0.05$; Fig. 3c).

Bivariate analyses illustrated a clear relationship between leaf N and photosynthesis for legumes (Fig. 4a), to the extent that leaf N_{area} explained almost half the variation in A_{sat} for these crops. However, and despite decades of breeding for increased yield, N_{area} was unrelated to A_{sat} for cereal and nonlegume dicots (Fig. 4e). In addition, bivariate analyses also show that N_{area} was positively related to g_s for cereals (to the extent that g_s increased $1.4 \text{ mol m}^{-2} \text{ s}^{-1}$ for every additional gram of leaf N; Fig. 4f), whilst N_{area} was unrelated to g_s for legumes (Fig. 4b) and nonlegume dicots (Fig. 4f). As expected on the basis of the literature, the relationship between N_{area} and WUE_i was strongest and most consistent for the legumes (Fig. 4c). Whilst also significant for the nonlegume dicots (Fig. 4g), the majority of data points are clustered, with the strength of the relationship originating in large part from three outlying measurements. Surprisingly, while WUE_i was unrelated to N_{area} for cereal crops (Fig. 4g), $\delta^{13}\text{C}$ ‰ –

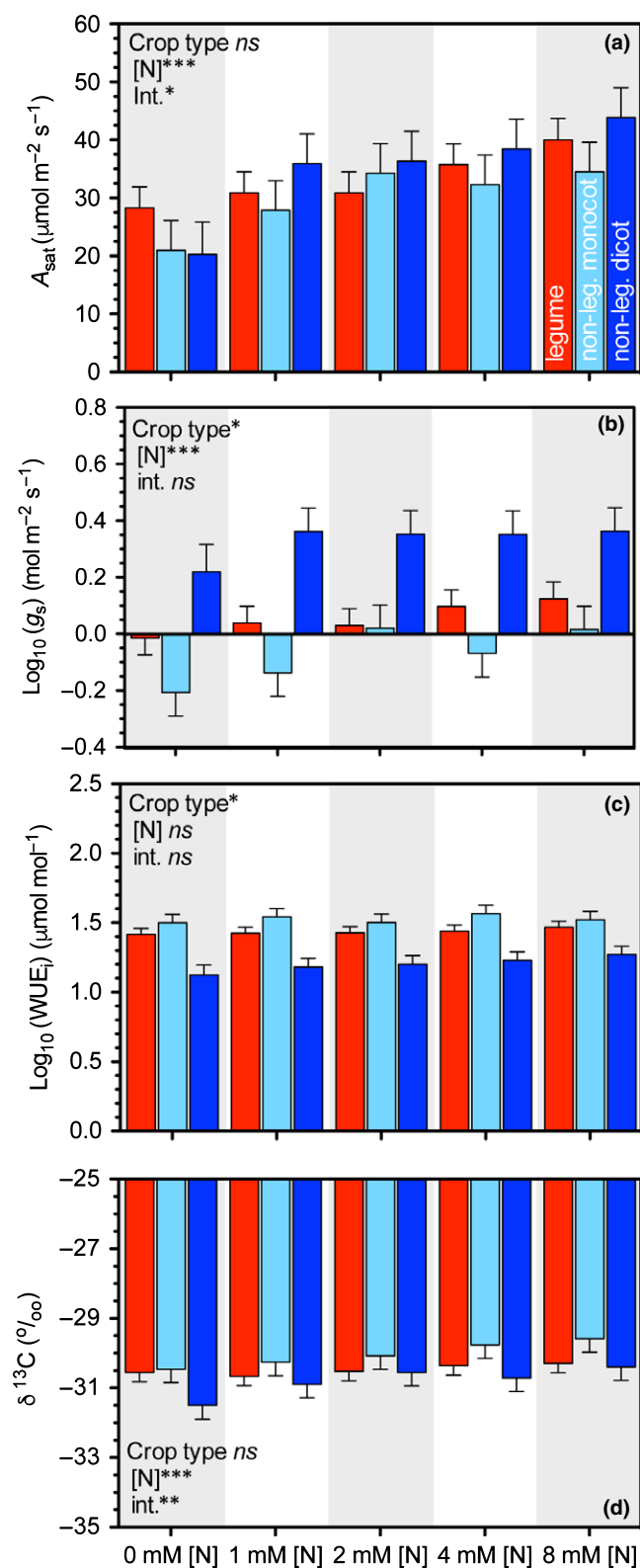


Fig. 3 Measures of leaf photosynthetic physiology for legume (red), nonlegume dicot (royal blue) and cereal (nonlegume monocot, light blue) agricultural crops from our controlled-environment comparison. (a) Light-saturated photosynthesis (A_{sat} , $\mu\text{mol m}^{-2} \text{s}^{-1}$); (b) stomatal conductance (g_s , $\text{mol m}^{-2} \text{s}^{-1}$); (c) intrinsic water-use efficiency (WUE_i , $\mu\text{mol CO}_2 \text{mol}^{-1} \text{H}_2\text{O}$); (d) carbon isotope discrimination ($\delta^{13}\text{C}$, ‰) of leaf tissue. g_s and WUE_i data were log_{10} -transformed before analyses to improve normality distributions. Data shown are estimated marginal means and SE for between five and seven replicates per species per treatment (see Supporting Information Table S1) from linear mixed models, with crop type, nitrogen addition rate ([N]) and their interaction as fixed effects and species as a random factor. ns, not significant ($\alpha = 0.05$); *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

point of clear distinction from nonlegume crop species, in which water loss via g_s , probably an unintended result of breeding, dominated the response to N_{area} (e.g. Fig. 4f). Our simulations show that legumes' greater demand for CO_2 via photosynthesis could provide large (2.5-fold) increases in WUE_i if legumes were instead bred for reduced g_s , that is, conservative water use, rather than for maximum A_{sat} (Fig. 5).

Discussion

Plant growth increased with the rate of N supply across all three functional groups, as is common for plant species used in agriculture around the world (e.g. Lawlor *et al.*, 2001). Plant N followed a similar pattern, increasing with every increase in the rate of N supply, across all functional groups and for each plant component (leaves, roots, stems). As we noted earlier, there were far stronger increases in plant N with the rate of applied N for non-legumes (than for legumes), which showed no sign of abating, even at the greatest rate of N application (8 mM) under the conditions of our experiment. Interestingly, while the concentration of externally applied N had a highly significant effect on leaf N concentrations, irrespective of whether they were expressed per unit area or per unit mass (Fig. 2), crop type (legume vs cereal vs nonlegume dicot) had no effect on N concentrations for any N application rate above zero – all species responded similarly, especially on a leaf area basis.

In a major review of crop photosynthesis, Richards (2000) stated clearly that for more than a century, there had been no increase in the rate of photosynthesis per unit leaf area for many crops, despite intensive breeding for yield. Richards went on to note that N fertilizer has been a quick and inexpensive substitute for genetic gains in photosynthetic capacity, that photosynthate production is mostly not limiting to growth and yield for the most intensively bred crops (e.g. cereals), and that, once acquired, N can be stored and used in a range of other metabolic processes of considerable benefit to the plant and to the crop.

Our results are in good agreement with both the conclusions of Richards (2000) and the more recent update provided by Makino (2011), who suggested that faster rates of photosynthesis in cereals like wheat or rice may be the result of breeding for cultivars with 'higher leaf N content depending on heavy N fertilization'. Makino (2011) drew upon the synthesis by Evans (1989) in noting that cereals also show far greater rates of photosynthesis than some other plant life forms (e.g. trees), and attributed this

considered a time-integrated indicator of WUE – was similarly related to N_{area} for both dicots and monocots, but unrelated to N_{area} for legumes (Fig. 4h). Enhanced Rubisco amount or activity could not be eliminated as a cause of enhanced WUE for legumes, as A_{sat} was strongly related to N_{area} (Fig. 4a). This is a

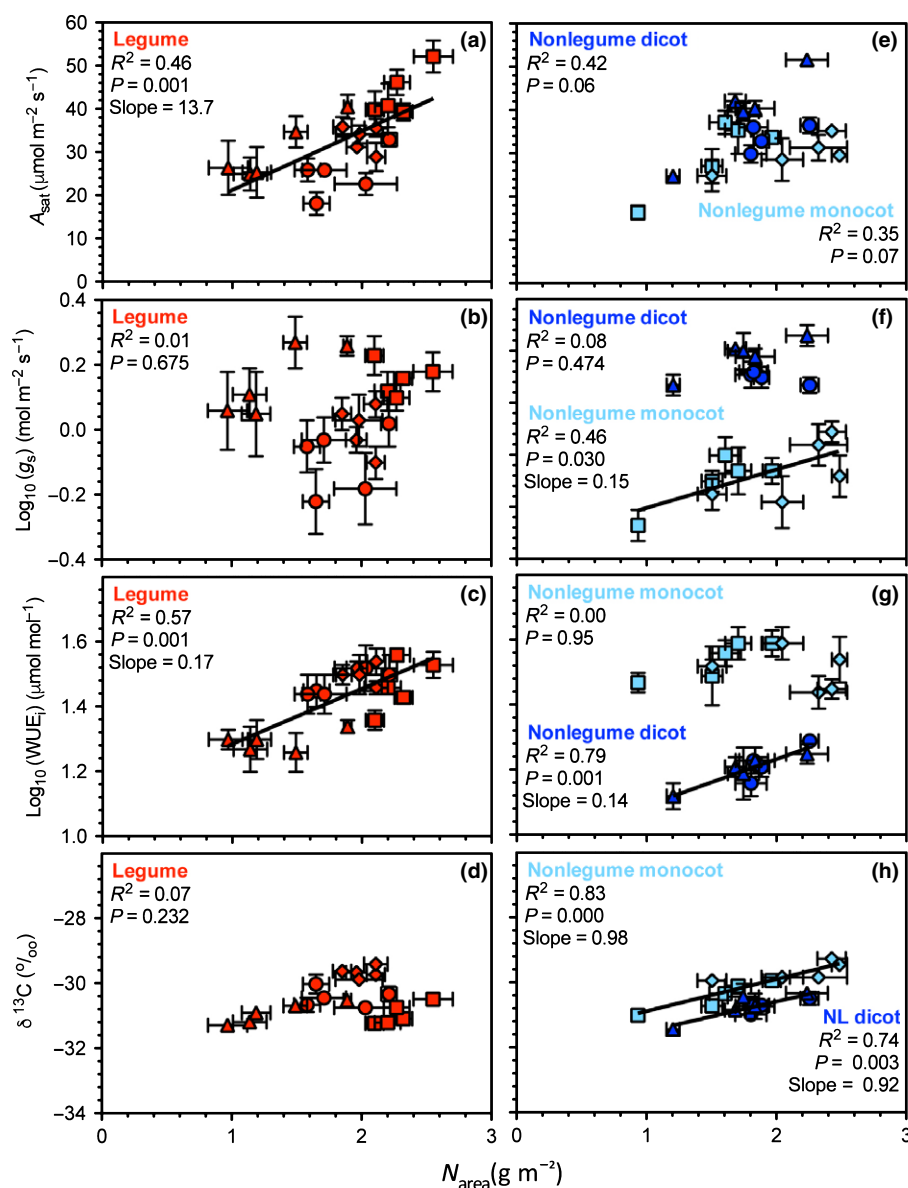


Fig. 4 Relationships between nitrogen per unit area (N_{area} , g m^{-2}) and light-saturated photosynthesis (A_{sat} , $\mu\text{mol m}^{-2} \text{s}^{-1}$) (a, e), stomatal conductance (g_s , $\text{mol m}^{-2} \text{s}^{-1}$) (b, f), intrinsic water-use efficiency (WUE_i , $\mu\text{mol CO}_2 \text{mol}^{-1} \text{H}_2\text{O}$) (c, g) and carbon isotope discrimination ($\delta^{13}\text{C}$ ‰) (d, h) for legume (red) (a–d), nonlegume dicot (royal blue) (e–h) agricultural crops from our controlled-environment comparison. g_s and WUE_i data were log_{10} -transformed before analyses to improve normality distributions. Data are means, with \pm SE as error bars for five to seven replicates per species per treatment (see Supporting Information Table S1). Statistics shown arise from Pearson correlations; slopes are shown for significant relationships only. Symbols are for legumes *Cicer arietinum* (squares), *Glycine max* (diamonds), *Lupinus alba* (triangles) and *Vicia faba* (circles); nonlegume dicots *Brassica napus* (circles) and *Helianthus annuus* (triangles); and nonlegume monocots *Hordeum vulgare* (squares) and *Triticum aestivum* (diamonds).

to greater allocation of N to Rubisco. The Evans (1989) dataset makes it clear that rates of carbon fixation by crops and annuals respond much more strongly to increases in leaf N than is the case for woody shrubs or trees.

In this context, the direct comparisons made here of the growth responses to N of legume and nonlegume crops, and the relationships among leaf N and photosynthetic and stomatal parameters, highlight several features that may help to guide future research and breeding programs. First, increases in leaf N with externally applied N are expected, and the much faster rate of accumulation of N overall for the intensively bred nonlegumes speaks to the consistency of patterns noted by Lawlor *et al.* (2001), Richards (2000) and Makino (2011). Breeding for yield under conditions of freely available soil N may have masked, or at least not fully revealed, N allocation traits that could still prove useful. Richards (2000), for example, noted that there remain periods of growth and specific attributes of wheat, such as stem elongation, that could benefit from increased (even temporarily)

supply of photosynthate. Second, leaf N was not a significant predictor of A_{sat} for these cereals and dicot crops (Fig. 4e). On the other hand, and in contrast to our hypothesis, which was motivated by observations in noncrop legumes (Adams *et al.*, 2016), leaf N and A_{sat} were very significantly related for the legume crops (Fig. 4a). As has been noted elsewhere, research and breeding programs for legumes are far less well established and less intensive than are those for cereals (Foyer *et al.*, 2016). The ability of legumes to acquire atmospheric N has probably played a role in reducing the attractiveness of research into legume photosynthesis. However, as shown clearly in Fig. 1, N fixation declines in significance as the supply of soil N increases, but the strong positive relationship between leaf N and A_{sat} remained linear and independent of the rate of supply of N to roots.

We do not presently know if the breeding of crop legumes generally, which is largely yield-focused, is on track to deliver optimum gains. Graham & Vance (2003) stated: ‘improvement in legume crop yields have not kept pace with those of cereals’. In

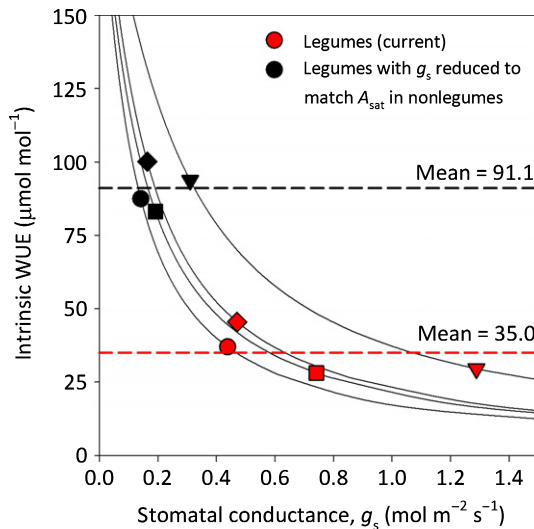


Fig. 5 Simulated gains in intrinsic water-use efficiency (WUE_i) that would result from breeding legumes with reduced stomatal conductance (g_s), such that light-saturated photosynthesis (A_{sat}) was reduced by 23.3% – the amount by which A_{sat} was lower in nonlegumes than in legumes under zero added N in our experiment. Lines represent g_s vs WUE_i relationships observed for four legume species (circles, *Vicia faba*; diamonds, *Glycine max*; squares, *Lupinus alba*; triangles, *Cicer arietinum*).

the same vein, there has been only modest amount of recent research into the interactions of N and photosynthesis in crop legumes (and, by definition, further interactions with the process of N fixation), and much of that which has been done focused instead on the effects of rising atmospheric $[CO_2]$ (e.g. West *et al.*, 2005; Leakey *et al.*, 2009). There are a number of contributing lines of research that have some bearing, including the greater carbon cost (and greater sink strength) per unit yield of seeds for legumes (Munier-Jolain & Salon, 2005) and the ability of legumes to respond to (and recover from) highly varying light regimes (e.g. Soleh *et al.*, 2016). Both depend on readily available N, one of the primary advantages of legumes over cereals. This advantage is further enhanced by the large and N-rich seeds produced by many legumes, such as *V. faba* in this study. In many respects, the positive relation between leaf N and A_{sat} for legumes underscores their greater potential for genetic gains via greater knowledge of photosynthesis and postfixation carbon allocation than is the case for nonlegumes. Although the present study focused on herbaceous legumes, hardy perennial grain legumes such as *Cajanus cajan* (pigeon pea) also offer promise in this regard, and are well suited to marginal lands.

Striking in our results was the strong relationship between leaf N per unit area and WUE_i of legumes (and nonlegume dicots), compared with the lack of any relationship for cereals. This result contradicts suggestions that application of N fertilizer should increase the WUE of cereal crops (Zhang *et al.*, 1998; Farquhar *et al.*, 2002; Cabrera-Bosquet *et al.*, 2007) via increased leaf N, Rubisco and hence photosynthetic activity. Application of N fertilizers could increase WUE through other N-related means, but the significance and process(es) remain unclear.

Water-use efficiency has attracted considerable attention from both physiologists and crop breeders, given often water-limited

conditions for cropping and the general significance of consumption of fresh water by agriculture. As Lawson & Blatt (2014) commented: ‘Improving plant WUE and a plant’s ability to cope with reduced water availability is high on the scientific agenda.’ Gilbert *et al.* (2011) found that stomatal conductance was the major driver of measured variation in WUE_i among soybean genotypes but, on the basis of their data, suggested that it was feasible to breed for high photosynthetic capacity to compensate for slowing rates of photosynthesis as a result of lower stomatal conductance. This accords with much of the earlier discussion. In our study, the strong relationships between WUE and leaf N for legumes and dicot nonlegumes have different drivers. Stomatal conductance clearly drove the relation for dicot nonlegumes, while for legumes, A_{sat} was mostly responsible.

In light of these differences, we calculated maximum possible gains in legume WUE_i if breeding were redirected to reducing stomatal conductance and water loss, rather than further increasing photosynthetic rates. Simulations using our dataset (Fig. 5) suggest that such an approach could increase legumes’ water-use efficiency 2.5-fold, over and above legumes’ already superior WUE relative to nonlegume dicot crops. Furthermore, these enhancements are most pronounced under conditions of zero/low exogenous (anthropogenic) N inputs, so, despite the intrinsic tradeoff between photosynthetic water- and N-use efficiency (Field *et al.*, 1983), they could be realized at no cost to the N-use efficiency of exogenous N.

Collectively, our data and model suggest that current agricultural genotypes of cereals, legumes and dicot nonlegumes could benefit greatly from breeding focused on reducing stomatal conductance. Managing N in cropping systems, and by definition within crop plants, will remain important, given its economic and environmental costs. Population growth in the coming century will require a large increase in agricultural production, while reduced fossil fuel supply, changes in rainfall patterns, climate change, and the need to reduce nitrogenous pollution will all require that we use less N and water per unit yield than we do now. Hawkesford (2014) called for research to focus on efficient management of N and, in addition to supporting major reviews (e.g. Richards, 2000; Makino, 2011), the data presented here illustrate the ‘inefficiency’ of current agronomic practices for increased yield that has resulted from decades of abundant use of fertilizer N. Legumes offer intrinsic advantages over nonlegumes in this regard. Our results argue that putative benefits of legumes have been masked by relatively high rates of water use (stomatal conductance) as a result of breeding aimed at maximizing absolute carbon gain rather than the efficiency of water and/or N use. We recommend that continuing research should focus on directing breeding towards reduced water use in legumes, and exploring the generality of the trends uncovered here across a wider range of legume genotypes.

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Author contributions

M.A.A., T.L.T., N.B. and T.N.B. designed the study. T.L.T., W.T.S. and C.H.B. conducted the experiment, T.L.T. analyzed data and presented the data, and T.N.B. prepared Fig. 4. All authors discussed the results, and M.A.A. and T.L.T. wrote the paper.

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Additional Supporting Information may be found online in the Supporting Information tab for this article:

Table S1 Number of replicates

Table S2 AIC values from linear mixed models

Table S3 Plant biomass

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