



Chicory stimulates companion legume species to fix more biological nitrogen

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Abstract

Purpose Chicory (*Cichorium intybus*) scavenges more soil mineral nitrogen (N) than perennial ryegrass (*Lolium perenne*). A glasshouse study was conducted to test whether (a) the percentage of N derived from the atmosphere (%Nd_fa) by a companion legume differs when grown with chicory or perennial ryegrass, and (b) there is an optimal ratio of

non-legume:legume in a pasture mix that maximises biological N₂ fixation.

Methods Chicory or perennial ryegrass was grown as a monoculture, or in a mixture with either lucerne (alfalfa, *Medicago sativa*) or subterranean clover (*Trifolium subterraneum*) at 25:75, 50:50 or 75:25 ratio (non-legume:legume based on plant numbers). Monocultures of lucerne and subterranean clover were included as controls.

Results All treatments containing chicory extracted more mineral N from the soil than corresponding treatments containing perennial ryegrass. Subterranean clover %Nd_fa was greater than lucerne. Combining lucerne with chicory in a 50:50 or 75:25 mixture increased the efficiency of N₂ fixation by > 20%. Growing chicory with subterranean clover in mixtures of 50:50 or 75:25 resulted in the highest %Nd_fa for growth among all treatments ($P < 0.05$). However, the amounts of N₂ fixed by subterranean clover in the perennial ryegrass-subterranean clover mixture were similar to those in the chicory-subterranean clover mixture since dry matter accumulation from subterranean clover was higher when grown with perennial ryegrass.

Conclusion The %Nd_fa of legumes was greater when grown with chicory than perennial ryegrass. Chicory mixed with subterranean clover or lucerne in 50:50 ratios provided the optimum balance between legume dry matter yield and N₂ fixation.

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Introduction

Legumes substantially contributes to the nitrogen (N) economy and grain yield of cereal crops when grown in any of the following sequences: (a) after grain legumes in a cropping sequence (Angus et al. 2015; Peoples et al. 2017; Zhao et al. 2022), (b) together with grain legumes in the same growing season in intercropping systems (Bedoussac et al. 2015; Ladha et al. 2022), (c) following a legume-based pasture ley in mixed/phased farming systems (Angus et al. 2000; Angus and Peoples 2012; Fillery 2001), or (d) in association with legume catch crops or cover crops (Li et al. 2015; Reckling et al. 2022). Much of the N-benefits of legumes is often attributed to their capacity for biological N_2 fixation in symbiosis with the soil bacteria rhizobia (Angus and Peoples 2012; Ladha et al. 2022; Li et al. 2015), and in the case of Australian farming systems, pasture legumes are the dominant source of N contributing to dryland cereal production (Angus 2001; Angus and Grace 2017).

The quantity of N_2 fixed by pasture legumes is a function of the amount of dry matter accumulated, the N concentration of that dry matter, and the proportion of this N derived from the atmosphere (%Nd_fa). The %Nd_fa in turn is heavily influenced by the concentration of plant-available N, particularly NO_3^- -N in the soil (Carlsson and Huss-Danell 2003; Dear et al. 1999; Peoples et al. 2001, 2012). Typically for an annual legume species, such as subterranean clover (*Trifolium subterraneum*), the %Nd_fa ranges from 65 to 95% (mean 81%. Fillery 2001; Peoples and Baldock 2001; Unkovich et al. 2010). However, in the case of a perennial legume species, such as lucerne (alfalfa; *Medicago sativa*), the %Nd_fa is often slightly lower than annual species under Australian rainfed conditions, ranging between 40 and 80% (mean 60%. Peoples et al. 2012; Unkovich et al. 2010). This presumably reflects the more extensive root systems of lucerne accessing mineral N to a greater depth in the soil profile over a longer period of growth each year than annual legume species (Peoples et al. 1998, 2012).

Generally the %Nd_fa of a legume species would be expected to increase throughout the growing season as the soil mineral N pool is progressively depleted and the demand for N to support legume growth increases during spring (Sanford et al. 1995; Unkovich et al. 1994). Growing legumes in mixtures with non-legume species has also been shown to improve %Nd_fa of legumes in both rainfed pastures and intensive forage systems (Carlsson and Huss-Danell 2003; Dear et al. 1999; Peoples et al. 2012) and by grain legumes in intercropping systems (Bedoussac et al. 2015; Ladha et al. 2022) due to the continual consumption of available forms of soil mineral N from the rooting zone by the non-legume. Yet despite reports in the literature that some non-legumes may differ in their competitive ability to assimilate soil mineral N (e.g. Delogu et al. 1998; Høgh-Jensen et al. 2006; Pirhofer-Walzl et al. 2012), few if any previous studies have compared the impact of different non-legume species on the %Nd_fa of their companion legume within the one experiment.

Both Høgh-Jensen et al. (2006) and Pirhofer-Walzl et al. (2012) found that chicory (*Cichorium intybus*) recovered both soil and applied fertiliser N more rapidly and more efficiently than perennial ryegrass (*Lolium perenne*). Chicory is a relatively new perennial forage species to Australian agriculture. It is important to know what effect the introduction of chicory might have on the contributions of N_2 fixation by annual and perennial legume species in mixed swards to the N economy of the subsequent crops were it to be widely adopted into the pasture phase of Australian ley-farming systems (Dear et al. 1999). Findings from a field experiment at Cowra in central New South Wales (NSW) in Australia suggested that subterranean clover may be a more suitable companion legume species for chicory than lucerne, as subterranean clover is less competitive in summer for soil moisture and nutrients (Li et al. 2012). However, the optimal ratio of chicory to legume and the influence of the inclusion of chicory in a mixed pasture on inputs of N_2 fixation by either of these legume species has not previously been investigated nor have the effect of chicory or perennial ryegrass on legume %Nd_fa ever been directly compared.

The experimentation described here aimed to test whether (a) chicory differed from perennial ryegrass in its impact on the %Nd_fa of companion legume species, and (b) there is an optimal ratio of

non-legume:legume to be grown in a pasture mix to maximise inputs of fixed N, given chicory is a non-legume species. We hypothesised that, given its reputation for efficient N uptake in the field, chicory would increase %Ndfa of companion legume species compared with ryegrass.

Materials and methods

Soil

The soil was collected from the 0–150 mm depth of a Red Chromosol (Isbell and National Committee on Soil and Terrain 2021), located on the Charles Sturt University farm, Wagga Wagga in southern New South Wales (147°35'04"E, 35°06'5"S). The area was under a pasture comprising a mixture of annual broadleaf and grass species with little to no legume species present. Moist soil was passed through a 5 mm screen and mixed thoroughly using a cement mixer to obtain a homogenous media.

The soil had a pH of 6.0 (1:5 soil:0.01 M CaCl_2) and mineral N concentration of 19.2 mg kg^{-1} . The effective cation exchange capacity was 6.73 cmol (+) kg^{-1} with exchangeable Ca^{2+} , Al^{3+} , Mg^{2+} , Mn^{2+} , Na^+ and K^+ as 4.51, 0, 0.64, 0.01, 0.02, 1.56 cmol (+) kg^{-1} , respectively. The soil reached its field capacity at a gravimetric water content of 18% (g 100 g^{-1}). Subsamples were taken to determine the gravimetric moisture content to calculate the equivalent mass of oven-dried soil added to pots.

Pot setup

Each pot (240 mm diameter $\times$ 380 mm height) was lined with a plastic bag to create a closed system. The equivalent of 11.7 kg of oven-dried soil was added to each pot and packed to an approximate bulk density of 1.3 Mg m^{-3} . Pots were then watered to 90% field capacity with deionised water by weight. A basal fertiliser was applied evenly to the soil surface to ensure that all essential nutrients, except N, were non-limiting (Supplementary Table 1). Pots were weighed daily and watered with deionised water to maintain the soil at 90% field capacity throughout the experiment.

Treatments

Experimentation was designed so that chicory or perennial ryegrass was grown at proportions of 100, 75, 50, 25 and 0% with either lucerne or subterranean clover. Species ratios were based on a total of 8 plants per pot. For example, a chicory/legume mixture of 75% would have 6 chicory plants and 2 legume plants sown, where 100% represented monocultures of either chicory or perennial ryegrass, and 0% were monocultures of lucerne or subterranean clover. Each monoculture or mixed species treatment was replicated four times.

Seeds were imbibed for a 24-hour period at 20 °C in petri dishes under dark conditions. At sowing, approximately 16 imbibed seeds of the respective species were sown at a depth of 5 mm in each pot and covered with soil and placed in a naturally-lit and temperature controlled (25 °C day) glasshouse at the Wagga Wagga Agricultural Institute, Wagga Wagga, NSW, Australia in April 2010. A commercial peat inoculant (rhizobial strains WU95 for lucerne and WSM826 for subterranean clover) was applied to the soil surface on the day of sowing to all pots, regardless of whether sown to legumes or not, and then washed into the seed zone with 150 mL of deionised water. Seven days after germination seedlings were thinned to 8 plants per pot to provide the desired species mix and proportions.

Plant sampling and analysis

All pots were destructively harvested 16 weeks after sowing. Plant shoots were removed by cutting the plants with a scalpel at the soil surface. The roots were shaken free from the bulk soil which was then mixed and sub-sampled for further chemical analysis. Roots were then gently washed over a 1 mm sieve to remove residual soil. Shoots and roots from each pot were separated into individual species. The clean root samples were stored in the refrigerator for two days during which time the nodule number per plant for legume root systems were visually counted. Only active nodules, determined by internal pink coloration, were quantified for each individual legume plant.

All plant materials (root and shoot) were oven dried at 60 °C for a minimum of 48 h and weighed for dry matter yield (DMY). Plant samples were then ground through a 0.25 mm mesh using a Cyclotec

sample mill. The ground material was sub-sampled and further ground to a fine powder using a puck and ring grinder. Total N and the ^{15}N composition were determined by combustion in an automated Nitrogen and Carbon analyser interfaced with a 20–20 stable isotope mass spectrometer (Europa Scientific, Crewe, UK). The ^{15}N data were expressed as $\delta^{15}\text{N}$ or parts per thousand (‰) relative to the ^{15}N composition of atmospheric N_2 (i.e. 0.3663 atom% ^{15}N), as follows:

$$\delta^{15}\text{N} = \frac{1000 \times (\text{atom}\%^{15}\text{N}_{\text{sample}} - \text{atom}\%^{15}\text{N}_{\text{air}})}{\text{atom}\%^{15}\text{N}_{\text{air}}} \quad (1)$$

Estimates of %Ndfa were derived by comparing the ^{15}N concentration of the legume shoot against the non- N_2 fixing reference plant (perennial ryegrass or chicory) growing in the same pot with the legume species. In the case of the legume monocultures, the average $\delta^{15}\text{N}$ of perennial ryegrass and chicory shoots was used for the calculation of %Ndfa. The reference plant provided a measure of the isotopic composition of plant-available soil N and it was assumed that the reference plant and legume accessed the same soil N pool (Unkovich et al. 2008). The %Ndfa was estimated by using the following equation (Shearer and Kohl 1986):

$$\%Ndfa = 100 (x - y)/(x - c) \quad (2)$$

where x is the ^{15}N of the total N of the non-legume reference plant, y is the ^{15}N of the shoot N of the legume and c represents a measure of the isotopic fractionation that occurs during N_2 fixation. In the present study the value used for c was -0.68‰ (Unkovich et al. 2008).

The quantity of N_2 fixed was determined by the quantity of N accumulated by the legume and the proportion of N fixed. In the current study only the N accumulated in the shoots was used to calculate N_2 fixation. The quantity of fixed N_2 present in legume shoots at each harvest was calculated as:

$$\text{N}_2 \text{ fixed (g)} = \text{Shoot N (\%)} \times (\%Ndfa) \times \text{Legume shoot DM (g)} \quad (3)$$

To compare N_2 fixation for each treatment across legume species, the relative efficiency of N_2 fixation was calculated from the N_2 fixation and shoot DMY data and expressed as kg shoot N fixed t^{-1} legume shoot dry matter (DM) (Peoples and Baldock 2001).

Soil sampling and analysis

All bulk soil from each pot, excluding roots, was collected and sub-samples (10.0 ± 0.2 g) were taken for mineral N analysis. Briefly, 50 mL of 1 M KCl solution was added to each sub-sample and shaken for 1 h on an end-over-end shaker, then allowed to settle for 30 min. The soil extracts were then passed through an Advantec 5 C filter paper before storing at 4°C until mineral N analysis. The concentration of $\text{NH}_4^+\text{-N}$ (Crooke and Simpson 1971) and $\text{NO}_3^-\text{-N}$ (Henriksen and Selmer-Olsen 1970) of the filtered extract solutions was determined colorimetrically using a Perstorp Alpkem segmented flow autoanalyser. The $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentration of the soil were not separated; therefore NO_3^- concentrations reported refer to the sum of the $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentration of the soil.

An additional sub-sample of between 30 and 50 g from each pot was taken to determine gravimetric soil moisture content by drying in an oven at 105°C for 24 h. Soil gravimetric moisture content was used to calculate soil mineral N concentration on an equivalent oven dried soil basis.

Statistical analysis

Associations between the measured responses and factors generated by the experimental design were examined by fitting linear mixed models. Pasture mixture and mixture ratio were used as predictors to classify data for response variables of soil mineral N, nodule numbers, %Ndfa and N_2 fixation efficiency. Total DMY per pot and plant N concentration were analysed using the same model as above, but for mixed treatments, DMY and plant N concentrations were analysed by introducing separately an additional factor, plant species, to classify the data, where plant species represented perennial ryegrass, chicory, lucerne or subterranean clover. Bonferroni corrections were used for multiple comparisons between responsive variables. Within the text, statistically significant differences are reported at the 95% confidence interval. All analyses were conducted using Genstat 22th edition.

Results

Changes in soil mineral nitrogen

The soil mineral N contents measured under chicory treatments was substantially lower than that under

perennial ryegrass mixtures or legume monocultures at the end of the experiment (Fig. 1). Chicory and perennial ryegrass treatments depleted the available soil mineral N by 92% and 78%, respectively, during the 16 weeks of growth. Chicory grown with subterranean clover in 50:50 and 75:25 mixture had the lowest soil mineral N (9 and 7 mg N pot⁻¹, respectively); significantly lower than in the chicory monoculture (18 mg N pot⁻¹). By contrast, similar amounts of soil mineral N were detected for perennial ryegrass grown in monoculture (42 mg N pot⁻¹) and in mixtures with lucerne (42–50 mg N pot⁻¹) or subterranean clover (39–45 mg N pot⁻¹). Soil mineral N was the highest in the subterranean clover (67 mg N pot⁻¹) and lucerne monocultures (68 mg N pot⁻¹, Fig. 1). Total soil organic N did not vary significantly between treatments after 16 weeks (data not shown).

Shoot and root dry matter accumulation

Chicory and perennial ryegrass mixed with subterranean clover had significantly higher total shoot DMY compared to chicory and perennial ryegrass monocultures (Fig. 2A), but no significant difference was detected when mixed with lucerne for either species compared to its corresponding monoculture (Fig. 2B). Mixtures that had 50% or greater subterranean clover had the highest shoot DMY. Of the non-legume component, chicory produced more shoot DMY than perennial ryegrass when mixed with subterranean clover. The subterranean clover monoculture had double the shoot DMY of the lucerne monoculture (Fig. 2A and B). Averaged across all mixtures, subterranean clover represented 69% of the total pot shoot DMY when mixed with

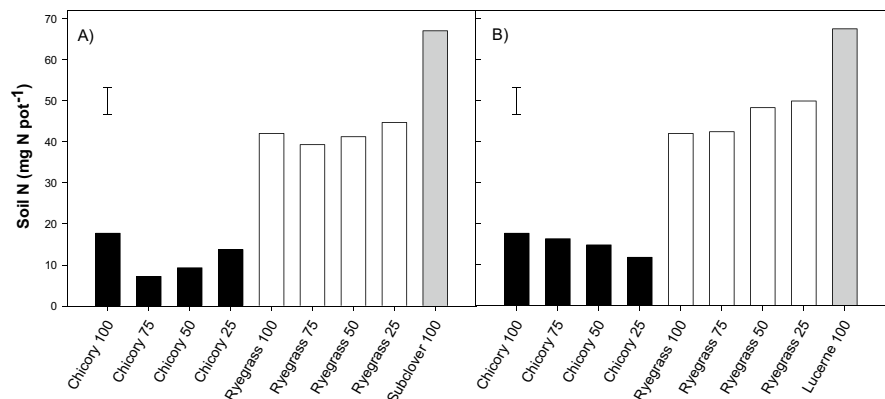
perennial ryegrass and 52% when mixed with chicory (Fig. 2A).

Mixtures containing 50% or more chicory had the greatest total root DMY (Fig. 2C and D). Perennial ryegrass and subterranean clover with 50:50 and 75:25 ratios had significantly greater total root DMY compared to the perennial ryegrass monoculture (Fig. 2C). Perennial ryegrass and lucerne mixtures with 25:75 and 50:50 ratios had significantly greater total root DMY compared to the perennial ryegrass monoculture (Fig. 2D). Chicory monoculture produced the highest DMY which was nearly twice as much as than other monocultures. There was no difference in root DMY between monocultures of perennial ryegrass, subterranean clover and lucerne. Mixing subterranean clover with either chicory or perennial ryegrass resulted in lower root DMY of subterranean clover compared to the subterranean clover monoculture. In contrast, mixing lucerne with chicory or perennial ryegrass reduced lucerne root DMY only in the 75:25 mixtures, but not in other mixtures compared to the lucerne monoculture. On average, root DMY of subterranean clover was 29% and 55% of total pot root DMY when grown with chicory or perennial ryegrass, respectively (Fig. 2C). As a comparison, root DMY of lucerne was 46% and 64% when grown with chicory and perennial ryegrass, respectively (Fig. 2D).

Nodule numbers and legume nitrogen fixation

The mean nodule numbers observed on subterranean clover root systems were more than double that observed for lucerne (Table 1). Subterranean clover nodule numbers were 10% higher in a 75:25

Fig. 1 Available soil mineral N remaining in the soil at week 16 for subterranean clover mixtures (A) and lucerne mixtures (B). Numerical values on the x axis labels refer to the proportion (%) of the indicated species. Vertical bars indicate L.S.D. at $P=0.05$



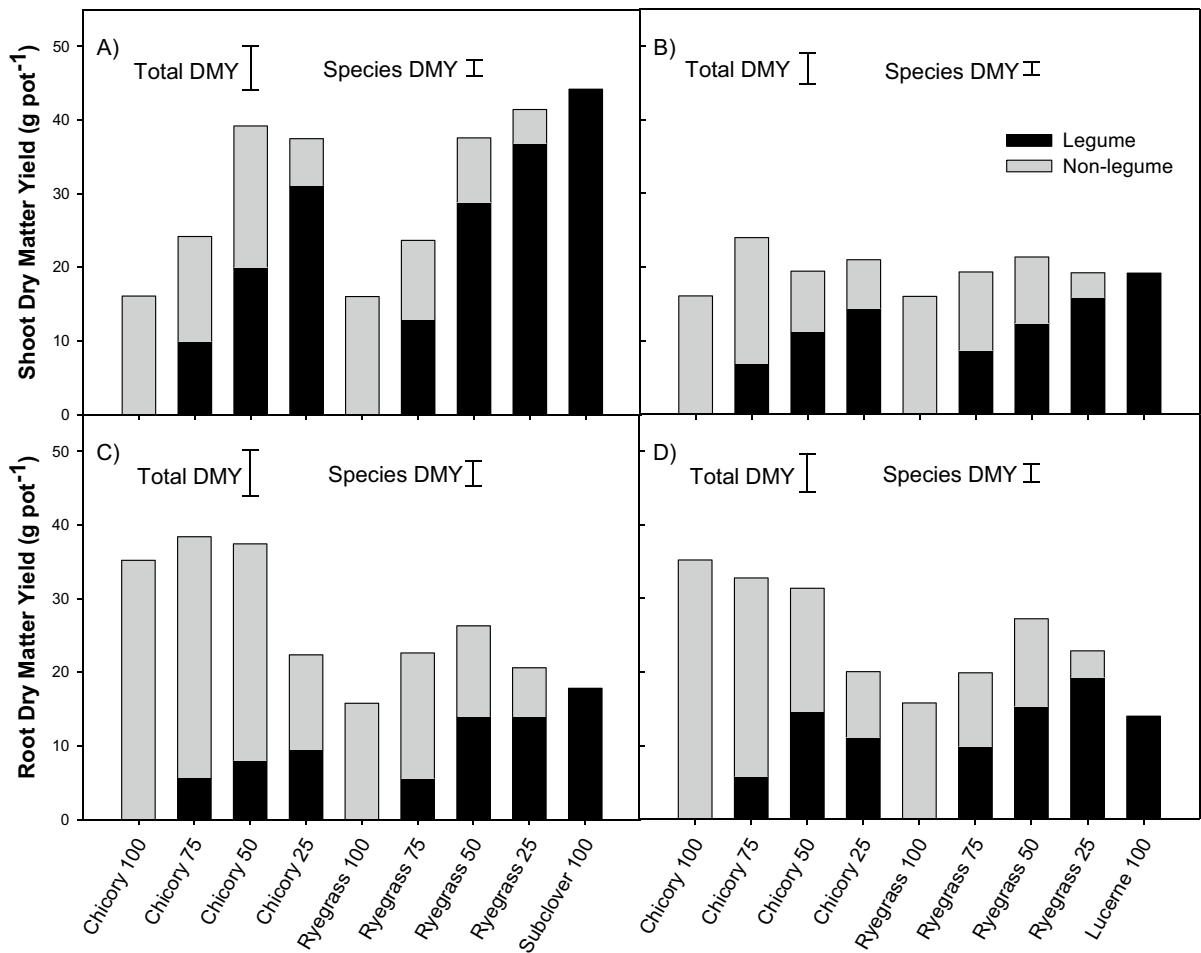


Fig. 2 Shoot (A, B) and root (C, D) dry matter yield (DMY) for chicory or perennial ryegrass mixtures with subterranean clover (A, C) and, lucerne (B, D). Vertical bars indicate L.S.D. at $P=0.05$

mixture of chicory:subterranean clover mixture than in a monoculture. All perennial ryegrass mixed with subterranean clover reduced the subterranean clover nodule number compared to the monoculture treatment (Table 1). There was no impact of mixtures on nodule numbers when perennial ryegrass mixed with lucerne except for the 25:75 mixture where nodule numbers on lucerne roots was 22% lower compared to the lucerne monoculture (Table 1).

Growing lucerne with either chicory or perennial ryegrass resulted in significantly higher %Nd_{fa} (85–95%) compared to the lucerne monoculture (79%). Similarly, growing subterranean clover with perennial ryegrass resulted in higher %Nd_{fa} (91–95%) compared to the subterranean clover

monoculture (84%; Table 1). Mixtures with chicory, regardless of ratio, resulted in close to total reliance on atmospheric N₂ for growth (98–99%; Table 1). The %Nd_{fa} of 50:50 and the 75:25 perennial ryegrass:subterranean clover mixtures (94–95%) were greater than calculated for the 25:75 perennial ryegrass:subterranean clover mixture (91%) and all mixtures of perennial ryegrass and lucerne (85–90%; Table 1). The 50:50 and the 75:25 chicory:luerne mixtures achieved similar %Nd_{fa} to the maximum levels observed for chicory and subterranean clover mixtures (Table 1).

The derived measures of N₂ fixation efficiency for lucerne and subterranean clover monocultures (equivalent to 29.5 and 27.3 kg N fixed t⁻¹

Table 1 Average nodule numbers per plant, percentage of shoot N derived from the atmosphere (%Ndfa), N₂ fixation efficiency (kg N fixed t⁻¹ legume shoot DM) for lucerne and sub-

terranean clover grown in various mixtures with either chicory or perennial ryegrass

Non-legume	Proportion (%)	Nodules plant ⁻¹		%Ndfa		kg N fixed t ⁻¹ shoot DM	
		Lucerne	Subterranean clover	Lucerne	Subterranean clover	Lucerne	Subterranean clover
Monoculture	0	51 e	110 bc	79 f	84 e	29.5 de	27.3 e
Chicory	25	49 e	104 cd	92 cd	98 a	31.7 cd	33.2 bc
	50	52 e	117 ab	95 ab	99 a	35.6 ab	33.4 bc
	75	47 ef	121 a	96 ab	99 a	36.7 a	31.5 cd
Perennial ryegrass	25	40 f	99 d	85 e	91 d	28.7 e	32.1 c
	50	47 ef	98 d	90 d	94 bc	31.2 cd	32.1 c
	75	46 ef	101 d	90 d	95 bc	32.7 c	31.3 cd

Values designated with the same letter under each parameter are not significantly different ($P=0.05$)

shoot DM) and the 25:75 mixture of perennial ryegrass:lucerne (28.7 kg N fixed t⁻¹ shoot DM) were the lowest of all treatments (Table 1). No variation in N₂ fixation was observed between chicory or perennial ryegrass mixtures with subterranean clover (31.3–33.4 kg N fixed t⁻¹ shoot DM). However, combining chicory and lucerne in a 75:25 mixture raised N₂ fixation efficiency to 36.7 kg N fixed t⁻¹ shoot DM, representing a 10–28% increase compared to all subterranean clover mixtures and other lucerne mixtures with the exception of the 50:50 chicory:lucerne treatment, and a 21–24% increase compared to lucerne grown in monoculture. This was around double the impact on N₂ fixation efficiency achieved by the 75:25 mix of perennial ryegrass:lucerne (Table 1). The inclusion of any of the examined quantities of either chicory or perennial ryegrass in a mixture with subterranean clover significantly increased the efficiency of N₂ fixation over a monoculture of subterranean clover. However, the increase in N₂ fixation efficiency tended to be slightly, but not significantly, higher with the inclusion of chicory (31.5–33.4 kg N fixed t⁻¹ shoot DM) compared to perennial ryegrass (31.3–32.1 kg N fixed t⁻¹ shoot DM).

Plant nitrogen accumulation

Measures of total plant (i.e. shoot+root) N for chicory grown as a monoculture (0.38 g N pot⁻¹) or in combination with legumes (0.27–0.67 g N pot⁻¹) were consistently greater than perennial ryegrass

monocultures (0.27 g N pot⁻¹) or the equivalent treatments containing a legume (0.09–0.27 g N pot⁻¹; Fig. 3). The subterranean clover monoculture had the greatest total plant N yield (1.91 g N pot⁻¹), almost double that measured for the lucerne monoculture (1.11 g N pot⁻¹; Fig. 3). Growing either chicory or perennial ryegrass in a 75:25 mixture with subterranean clover significantly reduced plant N yield compared with other subterranean clover mixtures. Similarly, a 75:25 mixture of either chicory or perennial ryegrass with lucerne significantly reduced plant N yield compared to other lucerne mixtures (Fig. 3). The greatest contribution of a non-legume to total plant N came from a 50:50 mixture of chicory with subterranean clover where the chicory component accumulated more plant N than chicory or perennial ryegrass in monocultures and all other mixture combinations with lucerne or subterranean clover. Growing chicory in a 75:25 mixture with either subterranean clover or lucerne also significantly increased chicory plant N compared to the chicory monoculture. No significant impact on perennial ryegrass plant N yield was observed when grown in any mixture combination with either subterranean clover or lucerne except in a 25:75 mixture where perennial ryegrass plant N was significantly less than other mixtures (Fig. 3). As the proportion of legume component (lucerne or subterranean clover) in mixture with chicory or perennial ryegrass decreased, total plant N yield also declined significantly as the contribution of N from either the lucerne or subterranean clover in the mixture was reduced.

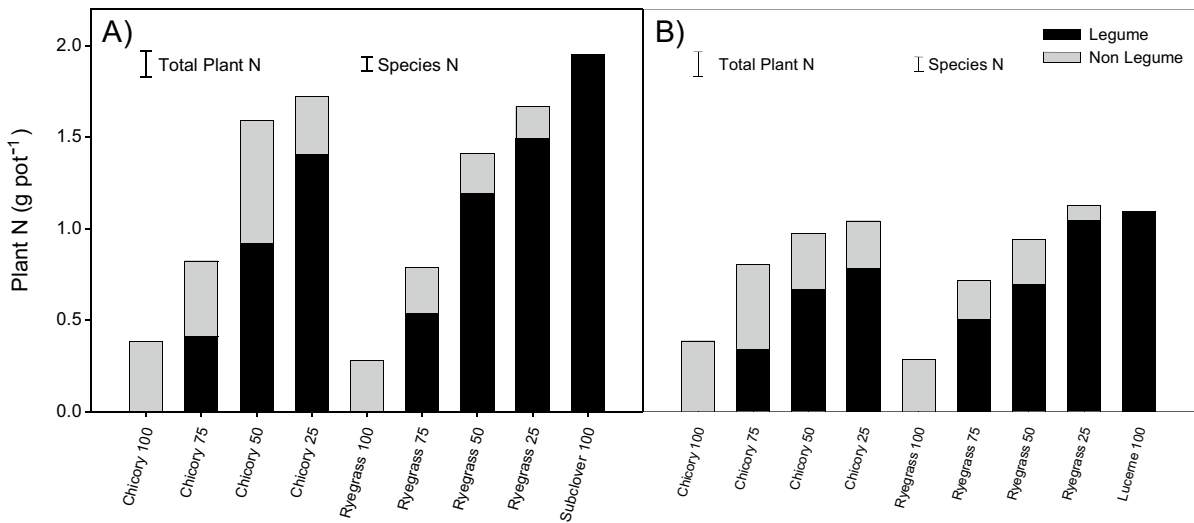


Fig. 3 Plant N (g pot⁻¹) for a combination of shoots and roots with various mixtures of chicory or perennial ryegrass with either subterranean clover (A) or lucerne (B). Vertical bars indicate L.S.D. at $P=0.05$

Discussion

Superior capacity of chicory to deplete soil mineral nitrogen

This study clearly demonstrated the superior capacity of chicory to deplete soil mineral N reserves compared to perennial ryegrass. All chicory treatments had lower residual soil mineral N contents at the end of experimentation and accumulated greater amounts of total plant N than the corresponding perennial grass treatments. The enhanced depletion of soil mineral N is consistent with the findings of Pirhofer-Walzl et al. (2012) who reported that chicory recovered 40% more N than a perennial ryegrass monoculture in an experiment investigating ¹⁵N uptake from 0.40, 0.80 and 1.20 m soil depths. Pure stands of field-grown chicory have also been shown to absorb 35–50 kg more N ha⁻¹ year⁻¹ than pure stands of perennial ryegrass under unfertilised and fertilised management regimes (0 vs. 120 kg fertiliser-N ha⁻¹ year⁻¹) (Hogh-Jensen et al. 2006). In another study comparing potential catch crops, chicory was reported to deplete soil nitrate levels by a further 21% compared to fodder radish (*Raphanus sativus*), sorrel (*Rumex acetose*) and perennial ryegrass (Askegaard and Eriksen 2007).

Suitable companion species with chicory

The current study indicated that mixtures of chicory (50% or greater) with subterranean clover increased soil mineral N uptake compared to a chicory monoculture, which inferred that interspecies competition facilitated greater mineral N uptake. Hogh-Jensen and Schjoerring (1997) reported similar observations whereby mixtures of white clover (*T. repens*) and perennial ryegrass absorbed higher amounts of soil N than pure stands of the either species. In another study, mixtures of two or four pasture species were shown to deplete soil mineral N reserves to a similar extent but increased N uptake by 92% and 96%, respectively, compared to a monoculture of any species (Pirhofer-Walzl et al. 2012). The differences in plant growth rate (hence plant N demand) and/or in N uptake kinetics will either inhibit or promote N uptake in a competitive situation (Hogh-Jensen and Schjoerring 1997). Interestingly, the greater depletion of soil mineral N in this study was not observed with mixtures of chicory and lucerne, or perennial ryegrass mixtures with either lucerne or subterranean clover. This suggests that the interspecies competition between chicory and subterranean clover was greater than occurred with lucerne mixtures. No doubt the greater root DMV detected in the 50:50 and 75:25 chicory:subterranean clover mixtures contributed to

the low soil mineral N observed in these particular treatments.

Depletion of soil mineral N by both non-legume species resulted in greater nodulation by subterranean clover, higher N_2 fixation efficiency for lucerne, and enhanced %Nd_{fa} by both lucerne and subterranean clover as the non-legumes became a progressively higher proportion of the sward composition. The %Nd_{fa} values obtained for subterranean clover in this study (83 – 99%) were within the range reported by other researchers for field-grown plants in Western Australia (Bolger et al. 1995; Sanford et al. 1995), and southern NSW and Victoria (Dear et al. 1999; Peoples et al. 1998, 2001). The %Nd_{fa} for lucerne (79 – 96%) was also comparable to the upper range observed in the field elsewhere in Australia, Europe and North America (Carlsson and Huss-Danell 2003; Hossain et al. 1996; McCallum et al. 2000). However, the measured lucerne %Nd_{fa} in this study were relatively high compared with lucerne field experiments previously undertaken in the same soil type in southern NSW (39–71%, Dear et al. 1999; Peoples et al. 1998). This may have been due to the lucerne plants being grown in a closed pot system, or simply reflect the short duration of the glasshouse study.

Nonetheless, despite the high %Nd_{fa} in the legume monocultures (79% and 83% for lucerne and subterranean clover, respectively), both %Nd_{fa} and the derived estimates of N_2 fixation efficiency were further enhanced wherever they were grown in association with non-legume species. Chicory increased the dependence of subterranean clover and lucerne on N_2 fixation to a greater extent than perennial ryegrass. While the effect of grasses on %Nd_{fa} has been described before (Carlsson and Huss-Danell 2003; Dear et al. 1999; Hogh-Jensen and Schjoerring 1997; Peoples et al. 2001), the significance of the data presented here is that it represents the first quantification of the consequence of the greater exploitation of soil mineral N reserves by chicory than perennial ryegrass on %Nd_{fa} by their companion legume in mixed swards. It is clear from the data that legume %Nd_{fa} was higher in mixtures with chicory than with perennial ryegrass. In some respects these outcomes were surprising as plants with fibrous root systems, such as perennial ryegrass, would usually be expected to explore a greater proportion of the soil volume than tap-rooted plants like chicory (Hogh-Jensen et al. 2006). Pirhofer-Walzl et al. (2012) postulated that the

larger root biomass of chicory would allow for the greater storage of N compared to perennial ryegrass, which would enable greater N uptake from soil before subsequent shoot N requirements were met.

The inhibitory effects of soil mineral N and/or fertiliser N on N_2 fixation by forage legumes has been well documented (Carlsson and Huss-Danell 2003; Peoples et al. 1998, 2001; Unovich et al. 1997). Sanford et al. (1995) concluded from their field studies in Western Australia that where non-legumes were present at high densities in a pasture sward, subterranean clover may be driven toward appreciably higher dependence on N_2 fixation. It would be interesting to repeat this experiment under a higher starting soil N status where greater pasture production and competition from chicory or other non-legume species, lower proportional mass of the legume component and lower %Nd_{fa} values, particularly for lucerne, might be expected.

Efficiency of N_2 fixation with different ratio of non-legume and legume

While it is common-place to examine the effect of different ratios of non-legume:legume on total DM_Y and inputs of N_2 fixation where cereals or oilseeds are intercropped with grain legumes (Bedoussac et al. 2015), it is quite unusual to undertake an equivalent comprehensive investigation with various pasture mixes, and it is novel to directly compare the interactions of legumes with different non-legume forage species, in this case a grass or non-legume dicot. The relative efficiency of N_2 fixation expressed as kg N fixed t^{-1} legume shoot DM_Y was perhaps the more important parameter to consider as it has a major influence on the rate of accumulation of fixed N within a pasture system. The higher %Nd_{fa} of legumes contributed to greater N_2 fixation efficiency of lucerne and subterranean clover when grown with a non-legume species compared to the legume monoculture. The 50:50 and 75:25 mixtures of chicory:lucerne had the greatest derived measures of efficiency of N_2 fixation of all mixtures, indicating that lucerne's reliance on N_2 fixed from the atmosphere increased without compromising DM production. While it is known that the quantity of N_2 fixed in pastures is usually strongly related to legume biomass (Bolger et al. 1995; Carlsson and Huss-Danell 2003; Dear et al. 2003; Peoples et al. 1998, 2001; Unovich et al. 2010) and that non-legume species can influence N_2 fixation

of the companion legumes by affecting %Ndfa, legume shoot biomass and tissue N concentration (Dear et al. 1999), the current study provided some unique and new insights into interactions of different non-legume species with legumes. Chicory reduced subterranean clover shoot biomass on average by a further 14% compared to perennial ryegrass across the respective ratios. However, %Ndfa was slightly greater for subterranean clover grown with chicory compared to corresponding perennial ryegrass mixtures. Therefore, due to the compensatory effect between %Ndfa and shoot DMY there was no difference in N_2 fixation efficiency for subterranean clover when mixed with either chicory or perennial ryegrass. In contrast, lucerne mixed with either chicory or perennial ryegrass reduced lucerne shoot DMY to a similar extent, while %Ndfa for lucerne was higher when mixed with chicory. This, therefore, translated into a higher N_2 fixation efficiency. The effect of chicory on lucerne shoot DMY may have been reduced due to the more erect growth habit of lucerne compared to subterranean clover, making it more competitive at intercepting light.

Although lucerne had a greater N_2 fixation efficiency, the combined higher %Ndfa and plant DMY with either chicory or perennial ryegrass mixed with subterranean clover (50:50 or 25:75) resulted in between 40 and 65% more plant N being accumulated per pot compared to respective lucerne mixtures. The major difference was the proportions of non-legume N per pot, with perennial ryegrass representing an average of 18% compared to 34% for chicory. In some treatments (50:50 subterranean clover, 75:25 lucerne) growing chicory with a companion legume resulted in the chicory plants having a higher accumulation of N than measured in the chicory monoculture. This might arise from some potential transfer of leguminous N to the chicory occurring via: (a) the rhizodeposition of N in the legume rhizosphere derived from the release of N-rich exudates or the death, decomposition and turnover of roots and nodules (Hauggaard-Nielsen and Jensen 2005; Jalonen et al. 2009; Paynel et al. 2001; Wichern et al. 2008), (b) the direct transfer of legume N through common mycorrhizal networks (Martensson et al. 1998), or (c) root proliferation within N rich patches in the soil (Robinson et al. 1999). Alternatively, root competition for the available soil N may have been greater between neighbouring chicory plants than between chicory and companion legume which may have allowed greater uptake of soil N by individual chicory plants growing in a mixture than the monoculture (Ladha et al. 2022). There are few studies

that can assist in distinguishing between these two prospective explanations. Hogh-Jensen et al. (2006) reported that chicory did not co-exist well with neighbouring leguminous plants and under high N conditions chicory outcompeted legumes such as white clover for common resources. For example, Hogh-Jensen et al. (2006) found that growing monocultures of chicory or the inclusion of chicory into perennial ryegrass-clover mixtures increased N accumulation per unit area compared to ryegrass-clover mixtures. Similarly, Pirhofer-Walzl et al. (2012) reported that including chicory in pasture mixtures increased N accumulation in plant tissue, which was believed to be due to chicory's capacity to assimilate N from deeper (>0.80 m) in the soil profile. Interestingly, Pirhofer-Walzl et al. (2012) did not observe greater N accumulation in chicory when grown in a mixture with white clover and lucerne compared to a chicory monoculture.

The 50:50 mixtures of chicory or perennial ryegrass with lucerne or subterranean clover appeared to be the most appropriate balance in terms of legume DMY, %Ndfa, N_2 fixation efficiency and total N accumulation. Previously, Sölter et al. (2007) and Thomas (1992) identified that legume contents of 25–50% were optimum for animal production and N supply for pasture production. In our study, increasing the non-legume content above 50% limited legume DMY and hence restricted the potential for the accumulation of legume N and inputs of fixed N. Where legume content was greater than 50% the prospects for improved %Ndfa and N_2 fixation efficiency were reduced; particularly where lucerne was the legume species.

Conclusions

Chicory has a superior ability to extract mineral N from soil than perennial ryegrass. The data presented here demonstrated that this translated into a greater enhancement of %Ndfa by both lucerne and subterranean clover in pasture mixes with chicory. Improvement in N_2 fixation efficiency were also observed in lucerne when chicory was 50% or more of the pasture mix. Varying the ratios of legume:non-legume in mixtures greatly affected dry matter production and N accumulation when subterranean clover was the legume companion species. It was concluded that the 50:50 mixture of chicory or perennial ryegrass with lucerne or subterranean clover represented the best balance in terms of legume DMY, %Ndfa, N_2 fixation efficiency and total

plant N accumulation. The current study provided valuable and unique information which can be used to better integrate the relatively new species of chicory into pasture mixes as a productive and N-efficient forage species that does not compromise the N contributions of legumes in Australian ley-farming systems.

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Data Availability The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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