

Article

Nitrogen Fixation and Resource Partitioning in Alfalfa (*Medicago sativa* L.), Cicer Milkvetch (*Astragalus cicer* L.) and Sainfoin (*Onobrychis viciifolia* Scop.) Using ^{15}N Enrichment under Controlled Environment Conditions

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Abstract: Availability of nitrogen (N) limits pasture production. Inclusion of legumes into grass pastures can provide an alternative N source through biological N_2 fixation (BNF), and enhance retention and cycling of soil C and N. Despite the use of alfalfa (*Medicago sativa* L.), cicer milkvetch (*Astragalus cicer* L.) and sainfoin (*Onobrychis viciifolia* Scop.) in grass-legume pastures to improve forage quality, relative BNF potentials and resource partitioning are unknown. We quantified BNF using ^{15}N isotope dilution and estimated resource partitioning in alfalfa, two cultivars of cicer milkvetch and two cultivars of sainfoin under controlled conditions. Percentage of nitrogen derived from atmosphere followed the order alfalfa (92%) > cicer milkvetch (87%) > sainfoin (81%); corresponding to estimated N contributions of 200, 128 and 65 kg N ha⁻¹ yr⁻¹, respectively, based on total herbage. Root dry matter was 24% to 36% greater than shoot dry matter in all of the legumes, providing substantial below-ground C and N. Cultivars of the same species did not differ in any measured parameter ($p > 0.05$). Despite the lower BNF in cicer milkvetch and sainfoin compared to alfalfa, their use may not negatively affect stand productivity and C storage.

Keywords: biological nitrogen fixation; pasture systems; ^{15}N dilution; resource partitioning; herbage yield; alfalfa; cicer milkvetch; sainfoin

1. Introduction

The productivity of pasture systems is often limited by the availability of nitrogen (N), and there is increasing interest in production systems that rely less on synthetic N fertilizer use [1]. Therefore, evaluating the potential of alternative sources of N is essential in maintaining N fertility of grazed pastures. Including legumes in grass-based pastures can help maintain high forage production by adding N through biological nitrogen fixation (BNF). The amount of N_2 fixed in grass-legume pastures ranges from 13 to 682 kg N ha⁻¹ yr⁻¹ [2], with about 80% transferred from legumes to grasses depending on the donor and recipient plant species [3]. For significant BNF to occur, an appropriate *Rhizobium* species must nodulate the legume. Biological N_2 fixation rates vary under different site and environmental conditions. Chen et al. (2004) [4] reported that under field conditions, a wide range (40–153 kg N ha⁻¹ yr⁻¹) of BNF is expected in pasture legumes. This variation reflects the vigor of legume growth which is controlled by climate, soil water and nutrient supply, companion grasses, defoliation, presence of grazing animals, pests and diseases and their interactions [4].

The alfalfa (*Medicago sativa* L.)–*Sinorhizobium meliloti* association is one of the most efficient associations between N₂-fixing bacteria and legume plants [5]. Despite its high efficiency, the amount of N fixed through this association is variable across sites, locations, and management regimes. Alfalfa can fix 4–650 kg N ha⁻¹ yr⁻¹ across different environments under different managements. For example, Peoples and Baldock (2001) [6] reported BNF between 4 and 284 kg N ha⁻¹ yr⁻¹ in alfalfa in a review of studies conducted in different locations of Australia and North America. McCaughey and Chen (1999) [7] reviewed experiments in North America and found that for field-grown grass-alfalfa mixtures, estimates of BNF ranged from 70 to 250 kg N ha⁻¹ yr⁻¹. In the Mississippi River Basin, BNF in alfalfa was reported to be 152 kg N ha⁻¹ yr⁻¹ [8]. Under field conditions in eastern Canada, Burity et al. (1989) [9] estimated that alfalfa plants fixed an average of 93 kg N ha⁻¹ yr⁻¹ in the first year, 258 and 227 kg N ha⁻¹ yr⁻¹ in the second and third year, respectively. The differences in BNF among the three years occurred because of N-stress at the onset of the symbiotic process. In western Canada, Kelner et al. (1997) [10] reported BNF of 174 and 466 kg N ha⁻¹ yr⁻¹ for the first year and third year alfalfa, respectively.

Biological N fixation rates in alfalfa were also different depending on the frequency at which materials are harvested under hayed pasture systems. Alfalfa fixed between 120 and 250 kg N ha⁻¹ yr⁻¹ in 2 to 6 cuts per year in Minnesota [11] or between 200 to 650 kg ha⁻¹ yr⁻¹ in up to 7 cuts per year in Argentina [12].

Both cicer milkvetch (*Astragalus cicer* L.) and sainfoin (*Onobrychis viciifolia* Scop.) have BNF rates (130 and 160 kg N ha⁻¹ yr⁻¹) within the range of other forage legumes but below the rate in alfalfa (cited in [13]). However, because alfalfa, cicer milkvetch and sainfoin were grown in different years with varying environments in the reported studies, it is impossible to compare their BNF directly. Sainfoin forms symbioses with bacteria in the genera *Mesorhizobium*, *Rhizobium* and *Bradyrhizobium* [14]. Sainfoin can also be cross-inoculated by *Rhizobium* species isolated from sweet vetch (*Hedysarum* sp.), crown vetch (*Coranilla* sp.), and purple and white prairie clover (*Dalea purpurea* and *Dalea candida*) [15]. Furthermore, inoculating sainfoin with rhizobia isolated from three arctic legume species (*Astragalus alpinus*, *Oxytropis maydelliana* and *Oxytropis arctobia*) improved BNF during cold phases of the growing season [16]. For cicer milkvetch, it is recommended to inoculate with an appropriate strain of *Rhizobium leguminosarum* [17]. However, there are very few reports of BNF rates in cicer milkvetch. From a literature review, cicer milkvetch fixed up to 157 kg N ha⁻¹ yr⁻¹, with a typical range of between 40–65 kg N ha⁻¹ yr⁻¹ (cited in [13]).

Apart from the N benefits of legume inclusion in pasture systems, other benefits are non-N-associated. For instance, in a study under the temperate steppe grassland in China, the combination of 50% alfalfa and 50% grass in the stand resulted in the most significant economic and ecological benefit through both enhanced water and N availability [18]. Nyfeler et al. (2011) [19] also reported that 40 to 60% legumes in grass-legume combinations efficiently transformed N into biomass by stimulating N utilization from both symbiotic and non-symbiotic sources. The incorporation of annual and perennial legumes into existing grassland pasture improved N cycling efficiency, with the perennials having higher efficiency over the annuals under a humid temperate climate in central New York State, U.S.A. [20]. Grass-legume pasture systems also can play a crucial role in soil C storage, mainly from inputs from the extensive root systems [21]. Deep-rooted pasture species have the potential to sequester C in subsurface soil layers and consequently counteract anthropogenic CO₂ emissions [21]. In a study examining C footprints of durum wheat grown in rotation with different crops in Saskatchewan, legume-durum systems had a lower C footprint relative to rotations with cereals only [22].

Crop cultivars and *Rhizobium* strains both have significant influences on BNF. In forage legumes, though not always the case, increases in production of legume cultivars through breeding or selection leads to a similar increase in BNF, primarily where the percentage of nitrogen derived from the atmosphere (% Ndfa) is higher than 80% [2]. In mixed swards under sheep grazing, two white clover cultivars were compared, and it was found that the Kopu cultivar produced 14% more dry matter

and fixed 35% more N compared to the Huia cultivar (cited in [2]). In another study, Ledgard et al. (1990) [23] found that higher legume production in two white clover cultivars was associated with lower total N concentration in legume herbage, leading to no increase in BNF. These findings showed that selection of legumes for improved BNF in breeding programs should consider total N concentration and % Ndfa and not be based solely on production. In this study, we used cultivars that were developed with little focus on BNF. The Oxley II was bred to improve seedling vigor and dry matter yield while Mountainview was bred to enhance the persistence of sainfoin within an alfalfa stand [24,25].

Despite the widespread and successful use of alfalfa in pasture systems in western Canada, producers have renewed interest in cicer milkvetch and sainfoin partly due to their non-bloat characteristics [26]. Cicer milkvetch and sainfoin have been used within North America for forage quality improvement and pasture regeneration. However, their BNF potentials and the relationship between their BNF and C and N dynamics in pasture systems have not been determined. The objectives of the study were (1) to quantify BNF in alfalfa, two cultivars of cicer milkvetch and two cultivars of sainfoin using the ^{15}N isotope dilution technique, (2) to estimate above- and below-ground partitioning of N and plant dry matter among the legume species under controlled environment conditions.

We hypothesized that: (1) alfalfa would add more total N than cicer milkvetch and sainfoin; (2) BNF would not be influenced by differences in cultivar within the same species, and (3) that resource partitioning would differ among the test legume species.

2. Materials and Methods

2.1. Experimental Design and Treatments

The growth chamber experiment was conducted at the phytotron facility in the College of Agriculture and Bioresources at the University of Saskatchewan, Saskatoon, SK, Canada. Biological N fixation was estimated using the ^{15}N isotope dilution technique [27]. Meadow bromegrass (*Bromus riparius* Rehm.) was grown and used as the reference species. There were six treatments: one cultivar of alfalfa (Algonquin), two cultivars of cicer milkvetch (Oxley II and Veldt), two cultivars of sainfoin [Common and Mountainview], and the non- N_2 -fixing reference (meadow bromegrass var. Armada). The plants were grown in 7.5 L pots (22.9 cm diam, 21.6 cm height) with soil (0–15 cm depth) from the Western Beef Development Center's (WBDC) Termuende Research Ranch located 8 km east of Lanigan, Saskatchewan, Canada (51°51' N, 104°54' W). The site has been under alfalfa/meadow bromegrass from 1998 until 2015 when it was sod seeded to two cultivars of cicer milkvetch and two cultivars of sainfoin. The soil at the site is mainly Black Chernozem (*Typic Haplocryoll*). In this study, surface soil (0–15 cm) was obtained from an area of the paddocks dominated by meadow bromegrass. The soil was homogenized and then mixed with quartz sand in a ratio of 9:1 (soil:sand) to facilitate drainage and root/nodule retrieval. Baseline soil properties are provided in Table 1. Organic matter (OM) content was determined by the loss-on-ignition method [28]. pH and electrical conductivity (EC) were determined on saturated paste in deionized water [29] using a Beckman 50 pH Meter (Beckman Coulter, Fullerton, CA, USA) and an Accumet AP85 pH EC meter (Accumet, Hudson, MA, USA), respectively. Potassium (K), Calcium (Ca), Magnesium (Mg) and Sodium (Na) were determined on saturated paste using inductively coupled plasma-optical emission spectrometry (ICP-OES). Available phosphorus and available potassium were extracted using the modified Kelowna procedure [30]; phosphorus was colorimetrically determined at 880 nm, and potassium was determined at 770 nm by flame emission. Available sulphur and nitrate-N were extracted with 0.01M CaCl_2 [31]; sulphate in the extract was determined using ICP-OES while nitrate-N was colorimetrically determined at 520 nm. Particle size analysis (PSA) was done using the mini-pipette method [32].

Table 1. Physicochemical properties of the Black Chernozem (*Typic Haplocryoll*) soil used for the biological N₂ fixation study.

Property	Value
pH (saturated paste)	7.3
Electrical conductivity (saturated paste) (dSm ⁻¹)	1.6
Organic matter (%)	6.4
Available phosphate-P (mg kg ⁻¹)	59
Available potassium (mg kg ⁻¹)	570
Available nitrate-N (mg kg ⁻¹)	45
Available sulphate-S (mg kg ⁻¹)	15
Cations in saturated paste extract	
Potassium (K) (mg L ⁻¹)	86
Calcium (Ca) (mg L ⁻¹)	188
Magnesium (Mg) (mg L ⁻¹)	58
Sodium (Na) (mg L ⁻¹)	6
Particle Size Analysis: Mini-Pipette Method	
% Sand (2.0 mm–0.05 mm)	48.6
% Silt (0.05 mm–2 µm)	40.0
% Clay (<2 µm)	11.4
Texture	Sandy Loam

The treatments were arranged in a Randomized Complete Block Design (RCBD) with four replicates. All species were grown from certified seed in separate pots. Twenty seeds of each species were sown per pot. Available research-grade *Rhizobium* inoculum was applied to the seed of each of the legume species using a clay-based carrier for alfalfa and a peat-based carrier for cicer milkvetch and sainfoin. These were *Rhizobium meliloti* L. for alfalfa, *Astragalus spp.* for cicer milkvetch, and *Bradyrhizobium spp.* for sainfoin [33]. The inoculant application rates were 8.3 g per kg of seed for alfalfa and 7.6 g per kg of seed for cicer milkvetch and sainfoin. Following establishment (~two months after seeding), the plants were thinned to six plants per pot, and ¹⁵N-NH₄NO₃ solution (10-atom % excess) applied at a rate of 5 kg N ha⁻¹. The ¹⁵N enriched solution was prepared by dissolving 0.345 g ¹⁵N-NH₄NO₃ in 1.2 L of distilled water (1.2 mM of N). Fifty mL of the solution was applied to each pot around the rooting zone with the aid of a syringe. The application of the solution was made directly to the soil, avoiding contact with the herbage. The plants were watered as needed (approximately every two days) on a weight basis to maintain the soil at ~80% field capacity throughout the experimental period. The growth chamber had a light intensity of a maximum of 450 µmol m⁻² s⁻¹. The mean day/night temperatures were 21/18 °C with 12/12 h day/night and 14/10 h day/night rhythm for spring/summer, respectively. Relative humidity was at the ambient level. Neither fertilizer nor pesticides were applied to the plants.

2.2. Plant Sampling and Analysis

At the late reproductive stage of the plants (~130 days after ¹⁵N application), the plants' shoots were harvested 0.5 cm from the soil surface. Roots and nodules for N₂-fixing plants and roots for the non-fixing plants were washed from adhering soil. Due to the size and numbers of nodules, it was not feasible to count the total number of nodules for the legumes. About 5 mg of the nodules from the legumes in each pot was removed from the roots to assess ¹⁵N enrichment. The remaining nodules were retained on the roots and contributed to the estimated root BNF. There were no differences in ¹⁵N enrichment between the nodule samples and the roots with nodules: only data for roots plus nodules are reported. All plant materials were oven-dried at 60 °C in a forced-air oven for 48 h to determine dry biomass. The dried samples were ground using a Wiley mill (Thomas Scientific, Swedesboro, NJ), followed by subsequent grinding to a fine powder using a ball grinder (8000D Mixer/Mill, SPEX SamplePrep® LLC., Metuchen, NJ, USA). The plant samples were then weighed using a microbalance (Sartorius Microbalance, CPA2P, Bradford, MA, USA) and subsamples encapsulated

in 8×5 mm tin capsules for mass spectrometry analysis. Sample sizes for shoot were $2.0 \text{ mg} \pm 0.3 \text{ mg}$, and for roots were $3.3 \text{ mg} \pm 0.5 \text{ mg}$. The samples were analyzed for atom % ^{15}N , and N concentration on a Costech ECS4010 elemental analyzer (Costech Analytical Technologies Inc., Valencia, CA, U.S.A.) coupled to a Delta V Advantage mass spectrometer (Thermo Scientific, Bremen, Germany). The standard deviation of repeated measurements ($n = 12$) of laboratory standards was $\pm 0.15 \text{ ‰}$. The percentage of N derived from the atmosphere (% Ndfa) was estimated using Equation (1) from [27]:

$$\% \text{ Ndfa} = \left(1 - \frac{\text{atom } \% \text{ }^{15}\text{N excess legumes}}{\text{atom } \% \text{ }^{15}\text{N excess bromegrass}} \right) \times 100 \quad (1)$$

where atom % ^{15}N excess is the ^{15}N content of the sample minus the ^{15}N content of atmospheric N_2 (i.e., 0.36637) in the fixing and non-fixing crops.

The amount of the N fixed was determined based on (1) the percentage of the plant N derived from atmospheric N_2 , (% Ndfa) and (2) the amount of N accumulated in the N_2 -fixing plant as detailed by [34]:

$$\text{N fixed} = \frac{\% \text{ Ndfa}}{100} \times \text{Legume N concentration (g N pot}^{-1}\text{)} \quad (2)$$

where legume N concentration was determined using Equation (3) below:

$$\text{Legume N concentration} = \frac{\% \text{ N}}{100} \times \text{Dry matter yield (g pot}^{-1}\text{)} \quad (3)$$

Total N_2 fixation on a whole plant basis was estimated using Equation (4). The equation was used to calculate the ^{15}N in the entire plant as an average of the shoot and root ^{15}N weighted by the total N contents (mg) of shoots and roots according to [35]:

$$^{15}\text{N whole plant} = \frac{\sum(^{15}\text{N root} \times \text{N root}) + (^{15}\text{N shoot} \times \text{N shoot})}{\sum(\text{N root} + \text{N shoot})} \quad (4)$$

2.3. Statistical Analysis

Both % Ndfa and quantitative N fixation data were analyzed statistically using One-way ANOVA by the PROC MIXED procedure in SAS (Statistical Analysis System, Version 9.4 for Windows™; SAS Institute, Cary, NC, USA) with legume species/cultivar as a fixed effect factor and replicate as a random effect factor. Before ANOVA, normality of residuals was tested using the Shapiro-Wilk statistic and homogeneity of variances was tested using Levene's test. No data transformations were required, as residuals of all data were homogeneous and normally distributed. Following ANOVA, Tukey's Honestly Significant Difference (HSD) was used to separate treatment means when significant. The SAS macro pdmix800 was used to perform pairwise comparisons at a 5% level of significance [36]. The PROC CORR procedure in SAS was used to develop a Pearson correlation ($n = 4$) to determine the relationship between whole plant quantitative N fixation and shoot and root dry matter yield.

3. Results

3.1. Nitrogen Derived from the Atmosphere (% Ndfa)

The percentage of nitrogen derived from the atmosphere (% Ndfa) was significantly different among the legume species ($p < 0.0001$). Alfalfa fixed the highest amount of N_2 in the shoot and the whole plant compared to cicer milkvetch and sainfoin (Figure 1a,c). The % Ndfa was not significantly different between alfalfa and cicer milkvetch in the root, but both were greater than sainfoin. In the shoots, alfalfa had the highest % Ndfa (~93%); there were no differences in % Ndfa among the cultivars of cicer milkvetch and sainfoin. Alfalfa had between 2–11% and 11–13% greater % Ndfa compared to cicer milkvetch and sainfoin, respectively. Cicer milkvetch had between 2–9% higher % Ndfa compared to sainfoin in the whole plant component. Although not statistically significant, the Veldt cultivar of

milkvetch tended to have higher % Ndfa than the Oxley II in the root and the whole plant components (Figure 1b,c). Similarly, the Mountainview cultivar of sainfoin tended to have higher % Ndfa than the Common cultivar. Alfalfa had similar % Ndfa values across all plant components. However, both cicer milkvetch and sainfoin had higher % Ndfa in the roots than in shoots.

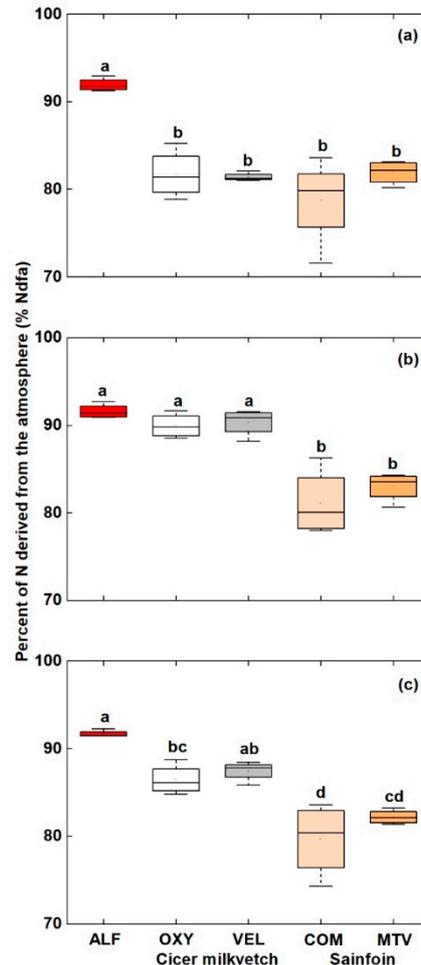


Figure 1. Nitrogen derived from the atmosphere (% Ndfa) in the shoot (a), root (b), and whole plant (c) among legume species and cultivars ($n = 4$) grown under controlled environment conditions. ALF = alfalfa, OXY = Oxley II milkvetch, VEL = Veldt milkvetch, COM = Common sainfoin, MTV = Mountainview sainfoin. The box is comprised of the 75th percentile, median, and 25th percentile, while the upper and lower whiskers are the maximum and minimum values, respectively. Boxes with the same letters indicate no significant differences among legume cultivars within a panel ($p > 0.05$) according to Tukey's HSD test.

3.2. Amount of N fixed

The amount of N fixed was also significantly different among the legume species ($p < 0.0001$), with alfalfa > cicer milkvetch > sainfoin (Figure 2a–c). Alfalfa had between 35–43% and 56–76% more N fixed compared to cicer milkvetch and sainfoin, respectively. The amount of N fixed was 27–63% more in cicer milkvetch compared to sainfoin. There was, however, no significant differences between the two cultivars of either cicer milkvetch or sainfoin in the shoot and whole-plant components. The Veldt cultivar had a significantly higher (29%) amount of N fixed than the Oxley II in the root (Figure 2b). There was no significant difference in the amount of N fixed between Mountainview and Common cultivars in all plant components.

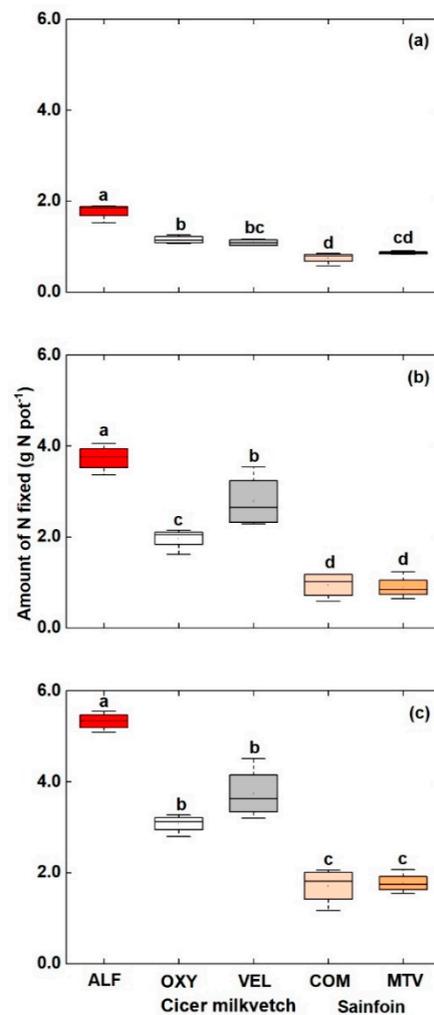


Figure 2. Amount of N fixed (g N pot^{-1}) in the shoot (a), root (b), and whole plant (c) among legume species and cultivars ($n = 4$) grown under controlled environment conditions. ALF = alfalfa, OXY = Oxley II milkvetch, VEL = Veldt milkvetch, COM = Common sainfoin, MTV = Mountainview sainfoin. The box is comprised of the 75th percentile, median, and 25th percentile, while the upper and lower whiskers are the maximum and minimum values, respectively. Boxes with the same letters indicate no significant differences among legume cultivars within a panel ($p > 0.05$) according to Tukey's HSD test.

There were no differences in root:shoot biomass among the difference species or cultivars, but significant differences were observed in root:shoot ratios of quantitative N among the legume species (Figure 3a,b). On average, alfalfa and cicer milkvetch had about twice as much N in their roots relative to their shoots, while sainfoin had similar amounts of N in roots and shoots (Figure 3b). The two cultivars of cicer milkvetch had significantly different root:shoot N ratio with Veldt having 33% more root N: shoot N than Oxley II. The Common cultivar of sainfoin tended to have higher (17%) root N: shoot N compared to Mountainview, although the difference was not statistically significant. Overall, all of the legume species had higher amounts of N fixed in the root compared to the shoot. Roots constituted up to 70% of total fixed N in alfalfa and cicer milkvetch and 53% of total fixed N in sainfoin.

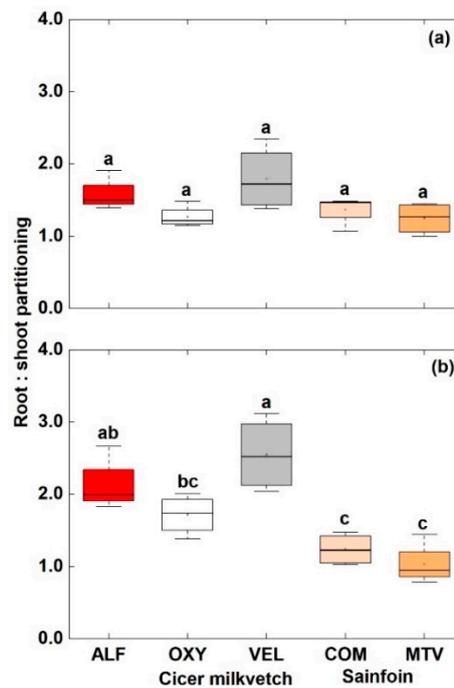


Figure 3. Root:shoot ratio in biomass (a) and quantitative nitrogen (b) among legume species and cultivars ($n = 4$) grown under controlled environment conditions. ALF = alfalfa, OXY = Oxley II milkvetch, VEL = Veldt milkvetch, COM = Common sainfoin, MTV = Mountainview sainfoin. The box is comprised of the 75th percentile, median, and 25th percentile, while the upper and lower whiskers are the maximum and minimum values, respectively. Boxes with the same letters indicate no significant differences among legume cultivars within a panel ($p > 0.05$) according to Tukey's HSD test.

3.3. Shoot and Root Dry Matter Yield

Shoot dry matter ($p < 0.0001$) and root dry matter ($p = 0.0008$) were different among the legume species. Following the pattern in N fixation, alfalfa had the highest dry matter yields compared to cicer milkvetch and sainfoin (Figure 4a,b). Alfalfa had 25% and 34% more root dry matter compared to cicer milkvetch and sainfoin, respectively. Root dry matter was 25% more in cicer milkvetch compared to sainfoin, and the Veldt cultivar of milkvetch had higher mean root dry matter relative to Oxley II. Despite the non-significant effects of root dry matter between Mountainview and Common cultivars, a trend of Common > Mountainview was observed.

Shoot dry matter followed the same pattern as root dry matter, with alfalfa having 26% and 34% more shoot dry matter yield compared to cicer milkvetch and sainfoin, respectively. Cicer milkvetch had 11% higher shoot dry matter compared to sainfoin. In contrast to root dry matter production, shoot dry matter yield tended to be slightly higher in the Oxley II compared to the Veldt. There was also a reverse trend Mountainview > Common between the cultivars of sainfoin in shoot dry matter, despite the non-significant effects (Figure 4a,b). There was a significant positive correlation ($p = 0.0167$; $r = 0.53$) between shoot dry matter yield and the total amount of N fixed (results not shown).

The partitioning of dry matter yield above- and below-ground among the legumes showed that more than half of the dry matter yield and associated N was allocated below-ground (i.e., root). Alfalfa and cicer milkvetch had a similar root: shoot ratio (1.6). Sainfoin, on the other hand, had a root: shoot ratio of 1.3 (Figure 3a). Again, despite the non-significant effects of legumes species and cultivars on root: shoot ratio, the partitioning tended to be higher (29%) in the Veldt compared to Oxley II. The pattern of the dry matter yield partitioning (root > shoot) suggests a substantial direct contribution of the root compared to shoot to both soil C and N in legume-based pasture systems, but with sainfoin allocating limited resources below-ground compared to the other two legume species.

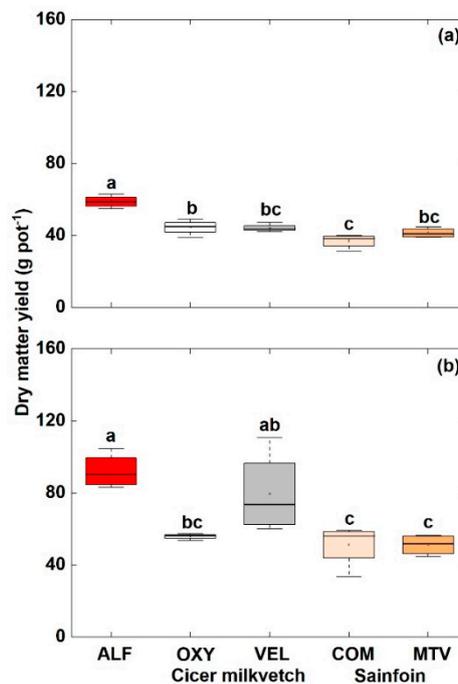


Figure 4. Shoot (a) and root dry matter yield (b) (g pot⁻¹) among legume species and cultivars ($n = 4$) grown under controlled environment conditions. ALF = alfalfa, OXY = Oxley II milkvetch, VEL = Veldt milkvetch, COM = Common sainfoin, MTV = Mountainview sainfoin. The box is comprised of the 75th percentile, median, and 25th percentile, while the upper and lower whiskers are the maximum and minimum values, respectively. Boxes with the same letters indicate no significant differences among legume cultivars within a panel ($p > 0.05$) according to Tukey's HSD test.

4. Discussion

4.1. Percentage of Nitrogen Derived from the Atmosphere (% Ndfa) and the Amount of N Fixed

Both % Ndfa and the amount of N fixed (g N pot⁻¹) in all plant components were significantly different among the legume species. Alfalfa had greater % Ndfa and total amount of N fixed compared to cicer milkvetch and sainfoin. This observation implies a high efficiency of the association between alfalfa and *Sinorhizobium meliloti*, contributing to higher BNF rates compared to cicer milkvetch and sainfoin. Oliveira et al. (2004) [5] found that the alfalfa-*Sinorhizobium meliloti* association is one of the most efficient associations between N₂-fixing bacteria and legume plants. The higher % Ndfa in alfalfa reflects the greater density of fibrous roots observed in the upper 20 cm of the soil, bearing most of the nodules for BNF.

The potential estimated amount of N fixed by alfalfa on an area basis (~200 kg N ha⁻¹ yr⁻¹) in this study is similar to rates reported in other studies in temperate regions of North America. In Minnesota, alfalfa fixed between 120 and 250 kg N ha⁻¹ yr⁻¹ after 2–6 cuts per year [11]. In western Canada, Kelner et al. (1997) [10] reported N₂ fixation of 174 and 466 kg N ha⁻¹ yr⁻¹ for the first year and third year of alfalfa, respectively.

The estimated N₂ fixation rate in cicer milkvetch in this study (128 kg N ha⁻¹ yr⁻¹) is comparable to values from other studies (cited in [13]). However, the N₂ fixation in sainfoin we obtained in this study (~65 kg N ha⁻¹ yr⁻¹) was significantly lower compared to reported rates (130–160 kg N ha⁻¹ yr⁻¹) (cited in [13]). The considerably lower amounts of N fixed in sainfoin compared to alfalfa and cicer milkvetch may reflect the inefficiency of the fixation process in sainfoin. Sainfoin was reported many years ago to be an inefficient N₂ fixer, showing N deficiency symptoms even in inoculated plants [15]. However, under field conditions in Switzerland either in a mixture or as a monocrop sainfoin fixed up to 197 kg N ha⁻¹ yr⁻¹ [37]. The lower N₂ fixation in sainfoin may also be because of the absence of

a companion legume with sainfoin. Cooper (1972) [38] reported that due to the capability of other legumes to supply sainfoin with N, sainfoin tends to contribute more to total yield when planted with other legumes. The higher energy requirement in sainfoin compared to alfalfa ($258 \text{ kg CH}_2\text{O ha}^{-1} \text{ day}^{-1}$ vs. $234.3 \text{ kg CH}_2\text{O ha}^{-1} \text{ day}^{-1}$ in alfalfa) contributes to lower BNF in sainfoin [39]. The higher requirement leads to smaller leaf area index and reduced usage of light energy and C fixation, indirectly resulting in lower BNF [39,40]. Despite the lower BNF by cicer milkvetch and sainfoin, their non-bloat characteristics and similar animal performance and nutritive value to alfalfa [26,41], make them viable alternatives to alfalfa for producers to use in grazing pasture systems. Roots were a significant component of the total fixed N in this study, contributing up to 70% of total BNF in alfalfa and cicer milkvetch but only 53% in sainfoin. This pattern is higher than reported in previous studies where roots constituted 30% and 30–40% of the total amounts of fixed N under field conditions in alfalfa/meadow bromegrass and clover/grass pastures, respectively [4,42]. Regardless, the legume roots can make a significant direct contribution to the N pool in pasture systems.

It is noteworthy that the proportion of plant N derived from BNF in alfalfa and sainfoin in this study are similar to typical values reported: 91% vs. 88% and 81% vs. 80%, respectively (cited in [13]). However, cicer milkvetch had higher BNF compared to reported values (87% vs. 70%) (cited in [13]). The higher BNF rate in this study may reflect the optimum conditions provided under controlled environment conditions. Under field conditions, a wider range of BNF is expected. The variations in BNF under field and controlled environment reflects the effects on the vigor of legume growth which is controlled by climate, soil water and nutrient supply, companion grasses, defoliation, presence of grazing animals, pests and diseases and their interactions [4].

4.2. Shoot and Root Dry Matter Yield

The shoot and root dry matter yields (g pot^{-1}) were significantly different among the legume species. Alfalfa had higher dry matter yield compared to cicer milkvetch and sainfoin. The similarity in the distribution of the dry matter yields among the legumes (root > shoot) is similar to that reported by [17]. It may be attributed to the difference in the rooting density among the legumes. The partitioning of dry matter (root > shoot) in all the legumes confirms the significant direct contribution of the below-ground plant component to soil organic matter, including C and N, in legume-based perennial pasture systems. This pattern follows observations of [43] who reported higher root turnover and rhizodeposition for perennial forage systems compared to annual crops, with a higher relative allocation of C below-ground. Fornara and Tilman (2008) [44] also reported that N_2 fixed by legumes, taken up and used by grasses in pasture systems, increases total below-ground biomass additions, serving as input for soil C and N.

The lower shoot and root dry matter yield in sainfoin compared to alfalfa is supported by other studies that reported that sainfoin produces approximately 5–20% less dry matter yield than alfalfa in western Canada [45,46]. The contributing factors to the lower dry matter yields and N input in sainfoin are mainly a lower leaf area indices (LAI), a less erect canopy structure and less efficient N_2 fixation [34]. The higher shoot dry matter yield in alfalfa relative to cicer milkvetch followed a similar pattern where cicer milkvetch yielded considerably less forage than alfalfa [46]. Though sainfoin generally produces lower yield and poorly competes, appropriate cropping management regimes can significantly contribute to the successful growth of sainfoin [47].

Alfalfa generally yielded more than cicer milkvetch, with yield ranging from 82 to 165% of cicer milkvetch yields [17]. Contrary to the results of this study, Lardner et al. (2019) [41] found no significant difference between alfalfa (A.C. Grazeland) and three cultivars of cicer milkvetch (Oxley, Oxley II and Veldt) in a two-year study. The trend in dry matter yield between alfalfa and cicer milkvetch is also supported by previous work where the yield in cicer milkvetch was generally about 75 to 85% that of alfalfa in western Canada [46–48]. However, in one study in the foothills region of western Canada situated in the Black soil zone, cicer milkvetch yielded more forage than alfalfa due to extensive damage to the taproots of alfalfa by pocket gophers [49].

Alfalfa has a deep taproot extending 7–9 m into the soil with fibrous roots concentrated in the upper 20 cm of the soil bearing most of the nodules. Sainfoin also has a deep taproot system with few main branches and twice as many fine lateral roots as alfalfa [50]. Cicer milkvetch has an aggressive creeping root system [50]. Despite the seemingly similar root architecture among the legume species, they differ in BNF. These findings suggest that breeding programs for forage legumes could consider factors other than only dry matter yield production [24–26].

The dry matter yields estimated from this study, conducted under optimal controlled environment conditions without pest pressure, confirms that alfalfa may still be a productive forage legume crop in western Canada [51,52] when compared to cicer milkvetch and sainfoin. The lower BNF in cicer milkvetch and sainfoin compared to alfalfa suggests that both cicer milkvetch and sainfoin may have lower potential to store soil C and N compared to alfalfa in pasture systems, though this assertion needs further research. However, in systems where N is not limiting, lower BNF and N input by cicer milkvetch and sainfoin may not have as significant an impact on the productivity of the system or soil C storage.

5. Conclusions

Biological nitrogen fixation and partitioning in alfalfa, cicer milkvetch and sainfoin are essential considerations when predicting the effects of legume species on C and N stocks and N fertility in pasture systems. The amounts of N potentially added by cicer milkvetch and sainfoin appear to be lower compared to alfalfa. Still, they may not reduce stand productivity and soil C storage in systems that are not highly N-limited. The higher root dry matter yield compared to the shoots in all the legumes species suggests a significant direct contribution of all the legume species and cultivars evaluated to soil C and N reserves. Additionally, other benefits of cicer milkvetch and sainfoin such as animal performance should also be considered in addition to their BNF potentials in determining their overall contribution to the productivity of pasture systems.

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