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ORIGINAL ARTICLE

Interseeded alfalfa N_2 fixation and transfer to maize are reduced by N fertilizer

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Abstract Establishing alfalfa (*Medicago sativa* L.) under a maize (*Zea mays* L.) silage companion crop is a promising approach for increasing forage production and profitability, but the role of biological N_2 fixation in this system has not been explored. This study utilized ^{15}N natural abundance techniques to assess biological N_2 fixation by interseeded alfalfa and transfer of fixed N to maize under three N fertilizer application rates. Across two locations in Wisconsin, USA, the results showed N_2 biological fixation supplied 59% of herbage N to interseeded alfalfa with no N fertilizer, though this was reduced to 15% when fertilized with 224 kg N ha^{-1}. Transfer of newly fixed N to maize was observed at both locations, with maize at harvest obtaining on average 28% of N uptake from fixed N without N fertilizer application, and 7% when fertilized with 112 or 224 kg N ha^{-1}. Additionally, both solo-seeded maize and tall fescue (*Schenodorus arundinaceus* (Schreb.) Dumort.) interseeded into maize system could produce appropriate reference crops for N_2 fixation and transfer calculations, provided the N-fertilizer strategies were similar to the interseeded alfalfa-maize system. Furthermore, results suggest the natural abundance method may not be effective if high rates of N fertilizer are applied at sites having inherently high levels of available soil N. The evidence for transfer of biologically fixed N_2 from interseeded alfalfa to maize offers interesting possibilities to leverage this process to ensure efficient N cycling, but it must be balanced against the need to ensure an adequate N supply for high maize silage yields.

Keywords Maize · Alfalfa · Intercropping · Inerseeding · Nitrogen · Fixation

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Introduction

Mixtures of multiple crop species hold the potential to enhance ecosystem services and maintain or improve crop production relative to single species by optimizing the utilization of limited resources, thus forming an important management option for ecological intensification (Bommarco et al. 2013; Malézieux et al. 2009). Intercropping grass and legume crop species, for example small grain crops with forage legumes, is a well-established mixed cropping strategy that can enhance crop production as well as ecological services compared to simpler rotations (Ofori and Stern 1987; Vrignon-Brenas et al. 2018). Interseeding alfalfa and maize silage is a promising forage production system that can provide several benefits of ecological intensification (Franco et al. 2021). In the interseeding system maize and alfalfa are planted simultaneously, with the interseeded alfalfa functionally serving as a cover crop by increasing living soil cover during and after maize silage production, then transitioning into full alfalfa production in subsequent years (Grabber 2016). Interseeding alfalfa and maize can reduce soil erosion and nutrient losses while increasing the overall forage yield and profitability compared to simple maize-alfalfa rotations (Osterholz et al. 2019, 2020, 2021a; Berti et al. 2021). Continued development of this cropping system could improve environmental and economic outcomes of forage production.

Nitrogen dynamics have long been recognized as an important factor in interseeded crop competition and crop productivity (Kurtz et al. 1952; Stern 1993). When planted in mixtures with legumes, several grass species including maize can acquire biologically fixed N from the legumes (Thilakarathna et al. 2016). A ^{15}N isotopic labeling study to conclusively demonstrated the transfer of N from alfalfa to intercropped maize (Shao et al. 2020). However, N transfer was reduced when N fertilizer was provided (Shao et al. 2020), and alfalfa N_2 fixation is typically reduced where soil N availability is high (Eardley et al. 1985; Cherney and Duxbury 1994). Recent work in the interseeded maize-alfalfa system has shown that interseeding alfalfa into maize increased the N fertilizer rate required for maximum maize yield compared with solo-seeded maize, suggesting that uptake of N by alfalfa may be an important competitive dynamic (Osterholz et al. 2021b). Additionally, soil N pools and maize yields in the interseeding system were shown to be positively related to N fertilization rate, further suggesting that N limitation can be reduced by increasing available N in the system (Osterholz et al. 2021a). As N fertilizer is needed to achieve high maize yields in the interseeded system, it is unclear to what degree interseeded alfalfa N_2 fixation may contribute to maize N nutrition under field conditions. Even so, the evidence of transfer of alfalfa N to maize suggests it may be possible to utilize alfalfa N_2 fixation to improve N availability in the interseeding system, thereby potentially increasing N cycling efficiency (Duchene et al. 2017).

Legume N_2 fixation is widely measured using ^{15}N stable isotope-based methods, and these approaches can also determine transfer of biologically fixed N_2 from legumes to intercropped non-legumes (Chalk et al. 2014). While several alternative ^{15}N approaches exist, the ^{15}N natural abundance technique has the advantages of being suitable for field studies, enabling estimation of fixation in the absence of fertilization, and not requiring expensive ^{15}N enriched fertilizer applications. The natural abundance technique is based on isotopic fractionation from natural N cycling processes that results in distinct ^{15}N signatures in actively fixing legumes and non-fixing plants. Specifically, newly fixed N in legumes is typically slightly depleted in ^{15}N (compared to atmospheric N), while N taken up from soil N pools is relatively enriched in ^{15}N due to accumulation of the heavier ^{15}N isotope through processes including nitrification and denitrification (Chalk et al. 2019). This approach has been successfully utilized in a number of different forage and grain crops (Peoples et al. 2015), but has not yet been implemented in interseeded maize-alfalfa systems. The natural abundance technique utilizes a non-N_2 fixing reference plant to establish the ^{15}N signature in the absence of N_2 fixation. The selection of the reference crop requires careful consideration of the similarity of the growth environment of the reference crop and crop of interest, particularly in regards to N sources and availability (Peoples et al. 2015).

In this study we explored the use of the ^{15}N natural abundance method to estimate N fixation by interseeded alfalfa and transfer of biologically fixed N to interseeded maize at three N fertilizer rates. Our hypothesis was that N_2 fixation by alfalfa and transfer to maize would be reduced by N fertilizer application due to the exogenous supply of plant available N suppressing fixation and transfer. In addition, we utilized two alternative reference crops to provide insight into best practices for future natural abundance studies in interseeded systems.

Methods

Experiment description

This study is the product of an experiment primarily designed to compare the N fertilizer response of maize interseeded with alfalfa to solo-seeded maize, partially described in Osterholz et al. (2021b). The experiment was conducted in 2017 at two locations with silt loam soils (fine-silty, mixed, superactive, mesic Typic Argiudolls) formed from glacial loess in southern Wisconsin, USA: the University of Wisconsin Arlington Agricultural Research Station (AARS) and the U.S. Dairy Forage Research Center Farm at Prairie du Sac (PDS). Previous work at these sites suggested the inherent soil N supply is typically somewhat greater at AARS than PDS (Osterholz and Grabber, unpublished data). Average annual precipitation and temperature at both locations is approximately 900 mm and 8 °C. Weather conditions in 2017 at both locations were generally conducive to maize production (Table S1).

The experiment consisted of three cropping systems (maize interseeded with alfalfa, maize interseeded with tall fescue (*Festuca arundinacea*, Schreb.), and solo-seeded maize) grown at three levels of N fertilization. Treatments were assigned to 3-m wide by 6.7-m long plots arranged in a randomized complete block design with three replicates at each site. The fixation of N_2 by alfalfa and its transfer to maize in the maize-interseeded alfalfa system were estimated by using solo-seeded maize and maize interseeded with tall fescue as reference crops. Four rows of maize (A6267, 102 d maturity, Agrigold, St. Francisville, IL) in all systems were no-till planted into plots on May 15 at AARS and May 5 at PDS using a 0.76-m row spacing. Immediately after maize planting, the three interrow areas of the interseeded systems were sown with four rows of alfalfa (55H94, Pioneer, Johnston, IA) or a turf type shade tolerant fescue (Falcon IV, Rutgers University) using a no-till drill with 0.165-m row spacing. The seeding rates were 79,000 seeds ha^{-1} for maize, 18 kg pure live seed ha^{-1} for alfalfa to ensure high seedling establishment (Grabber 2016), and 22 kg ha^{-1} for tall fescue based on seed company recommendations.

This study employed three N rates for the maize interseeded with alfalfa and solo-seeded maize: 0 kg N ha^{-1} (0N), 112 kg N ha^{-1} (112N), and 224 kg N ha^{-1} (224N). The maize with fescue system received only the 0N and 224N rates, so the only reference crop available at the 112N rate was solo-seeded maize. Urea treated with N-(n-butyl) thiophosphoric triamide (Agrotain, Koch Agronomic Services, Wichita, KS) was the fertilizer form, with half the N rate applied as a surface broadcast at planting and the other half side dressed along the maize row at the V5 maize development stage. In order to improve the likelihood of successful alfalfa establishment, prohexadione calcium (calcium 3-oxido-5-oxo-4-propionylcyclohex-3-enecarboxylate, Fine Americas Inc., Walnut Creek, CA) was applied to interseeded alfalfa when it reached ~ 0.3-m in height (Grabber et al. 2016; Osterholz et al. 2018). Maize was harvested for silage at 30–37% dry matter content (on September 12 and September 6 at AARS and PDS, respectively) using a forage plot harvester. Additional details of crop management are found in Osterholz et al. (2021b). The experimental plots were surrounded by a 6-m wide border of maize silage to uniformly shade interseeded alfalfa and tall fescue, and surrounding the maize border an additional 3-m wide border of solo-seeded alfalfa was planted at the same time and rate as interseeded alfalfa to provide an estimate of N_2 fixation by solo-seeded alfalfa.

Sampling and analysis

Plots were split into multiple sampling areas separated by undisturbed maize plants to facilitate destructive plant sampling while maintaining uniform shading of plot areas sampled at later dates. Interseeded alfalfa and tall fescue and the solo-seeded alfalfa check were not clipped or harvested during the growing season prior to plant sampling. Sampling was conducted four times to coincide with the V12 (July 17 and July 10 at AARS and PDS, respectively) and R1 (August 21 and August 14 at AARS and PDS) maize development stages, maize silage harvest (September 12 and September 6 at AARS and PDS) and in the late fall prior to a killing frost (November 7), hereafter referred to as July, August, September, and November sampling times. Interseeded maize and the solo-seeded maize reference crop were sampled on the first three sampling dates. Alfalfa aboveground growth had senesced by the time of R1 sampling due to shading by maize and thus was not sampled at R1 or at maize harvest, so alfalfa and fescue were sampled only on the V12 and late fall sampling dates. Maize samples at V12 and R1 consisted of the aboveground biomass of six representative whole plants taken from the middle-2 maize rows, while at maize harvest a subsample was taken of the material harvested from the middle-2 maize rows with a forage plot harvester. Alfalfa and tall fescue samples were collected by cutting above-ground growth from all four rows in a 0.23 m^2 area of the middle maize interrow. At each alfalfa/fescue sampling we also collected four samples of the solo-seeded alfalfa from four quadrants of the solo-seeded alfalfa border area; in November only the alfalfa re-growth following silage harvest was sampled. Plant samples were dried at 60 °C, weighed, and a subsample was finely ground. Subsamples were analyzed in duplicate for N concentration and $^{15}N/^{14}N$ isotope ratio using a coupled elemental analyzer (Carlo Erba, Milan, Italy)/isotope ratio mass spectrometer (PDZ Europa, Crewe, UK). Samples with duplicate discrepancies of > 0.01‰ ^{15}N were re-analyzed until two duplicates with agreement below that threshold was obtained. Dry weights of samples were converted to kg ha^{-1} by scaling up the g m^{-2} sample values for alfalfa or tall fescue, or by multiplying by the plant population for maize, which was estimated by counting stalks in the plots following silage harvest.

Calculations

Delta ^{15}N ($\delta^{15}N$) values of the samples were calculated as the deviation from atmospheric N_2, assumed to equal 0.3663 atom % ^{15}N (citation). Alfalfa N fixation was calculated by first calculating the proportions of N derived from atmosphere (Ndfa) as a percentage using the following mixing model:

$$\%\ \mathrm{Ndfa} = 100 \times \frac{\left(\delta^{15}\mathrm{N}_{\mathrm{ref}} - \delta^{15}\mathrm{N}_{\mathrm{alf}}\right)}{\left(\delta^{15}\mathrm{N}_{\mathrm{ref}} - \mathrm{B}\right)} \quad (1)$$

where $\delta^{15}N_{ref}$ is the ^{15}N signature of the reference crop (solo-seeded maize or fescue interseeded into maize), $\delta^{15}N_{alf}$ is the ^{15}N signature of the alfalfa, and B is defined as the $\delta^{15}N$ signature of alfalfa when dependent solely on N_2 fixation. Ndfa was calculated for each N rate in each experimental block, using the $\delta^{15}N_{ref}$ and $\delta^{15}N_{alf}$ values from the same N rate in a given block. For the 0N and 224N plots the % Ndfa was calculated twice using each available reference plant (e.g. for alfalfa with 224N the reference plants were interseeded fescue with 224 N and solo-seeded maize with 224N). We did not measure B in this study but assumed a B value of − 0.7 for alfalfa in the Midwestern USA (Blesh and Drinkwater 2012). Previous research has shown small variations in B values have relatively minor impacts on N_2 fixation calculations (Unkovich et al. 1994).

Similar to the alfalfa calculations, % Ndfa in maize interseeded with alfalfa was calculated for each N rate by the same mixing model as above, where $\delta^{15}N_{ref}$ represented the maize reference crop (either solo-seeded maize or maize with interseeded fescue), and $\delta^{15}N_{alf}$ was replaced by $\delta^{15}N$ signature of the maize interseeded with alfalfa (Peoples et al. 2015). As with the alfalfa, total N from fixation was calculated by multiplying % Ndfa by the total N content of the maize interseeded with alfalfa. The % Ndfa was multiplied by the N content of alfalfa or maize interseeded with alfalfa to calculate the total N fixed in kg N ha^{-1}.

Statistics

Differences in the $\delta^{15}N$ signatures were tested with three ANOVAs: one for maize samples across the three maize sampling dates (July, August, and September); one for alfalfa and fescue samples from the July and November sampling dates; and a combined analysis comparing maize, alfalfa, and tall fescue samples from the July sampling date. As with $\delta^{15}N$, additional ANOVAs were conducted to analyze % Ndfa for maize interseeded with alfalfa and interseeded alfalfa. Furthermore, ANOVAs were conducted separately for alfalfa and maize biomass, total N content, and total Ndfa. For each ANOVA, all N rate and cropping system combinations were considered as treatments, and treatments and locations were considered fixed effects. For the analyses that included multiple sampling dates, the date was considered a repeated measure at the plot level with an unstructured covariance structure. Block within location was considered a random factor. Separation of treatment means was by LSD with a significance level of $P < 0.05$. Finally, a paired *t* test was used to compare the % Ndfa estimates calculated using different reference crops, with estimates from the same plot paired. For all ANOVAs, plots of residuals were visually assessed for normality, and tests of homogeneity of variance were conducted for location and date. Analyses were conducted in SAS v. 9.4 (SAS Institute, Cary NC) and ANOVAs are summarized in Table S2. Main effects, interactions, and treatment differences described in the Results and the Discussion were significant at $P \leq 0.05$.

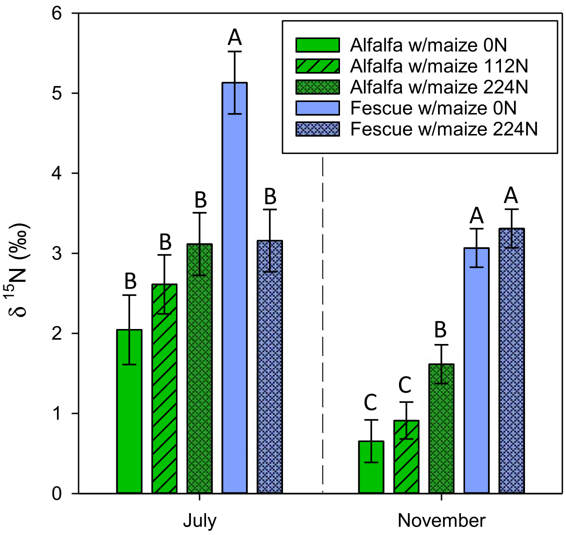

Fig. 1 Alfalfa interseeded with maize and fescue interseeded with maize δ^{15}N signatures in July and November. Three N fertilization regimes (0N, 112N and 224N) were applied. Results were averaged across the two locations. Different letters indicate differences among treatments within a date ($P < 0.05$) and error bars are standard error of the mean

Results

δ15N signatures

The δ^{15}N signatures of biomass samples differed by crop type (maize, alfalfa, and tall fescue) and were influenced by N fertilization regime and sampling time but were generally not influenced by location. Maize, alfalfa, and tall fescue samples were all collected at the July sampling date, and for the interseeded maize with alfalfa system the δ^{15}N of maize (4.5–5.1‰) was greater than alfalfa (1.9–2.2‰) at the 0N fertilizer rate. At both 112N and 224N fertilizer rates there was no difference in δ^{15}N between maize and alfalfa (Table S2; Figs. 1 and 2). Also, in July, for a given N fertilization regime the maize interseeded with tall fescue, interseeded tall fescue, and solo-seeded maize had δ^{15}N values that were within 0.7‰ of each other and not significantly different. Tall fescue had greater δ^{15}N than alfalfa at the 0N fertilizer rate in July and November, but at the 224N fertilizer rate the tall fescue had greater δ^{15}N than alfalfa only in November (Fig. 1). The δ^{15}N signature of the

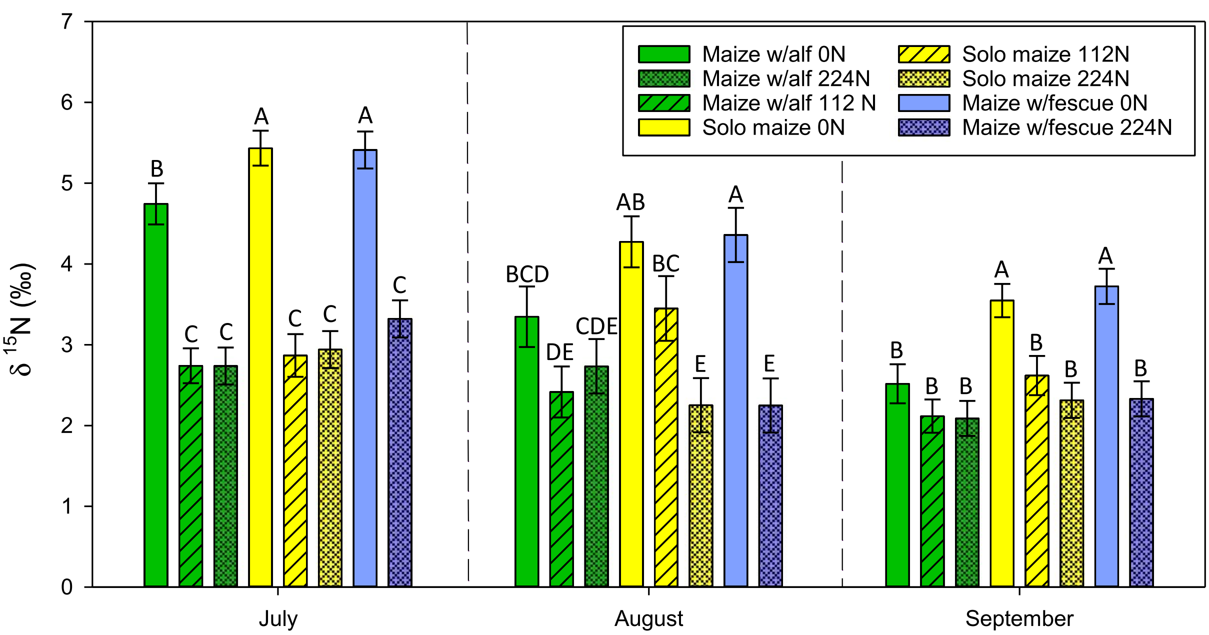

Fig. 2 Maize δ^{15}N signatures from maize interseeded with alfalfa, maize interseeded with fescue, and solo-seeded maize at three sampling times (July, August, September). Three N fertilization regimes (0N, 112N, and 224N) were applied. Results were averaged across two locations in the study. Different letters indicate significant differences among treatments within a sampling date ($P < 0.05$) and error bars are standard error of the mean

solo-seeded alfalfa check was numerically similar to 0N interseeded alfalfa in both July and November. Additionally, time of year was a significant determinant of $\delta^{15}N$, with a significant decline generally observed over the course of the season for each of the three crop types, although the 224N fertilized tall fescue and solo-seeded maize treatments both had similar signatures across the sampling dates.

Cropping system affected the $\delta^{15}N$ signatures of maize in a few cases that depended on the sampling time and N rate (Fig. 2). Differences by system were primarily observed for the 0N fertilizer rate, where in July and September the maize interseeded with alfalfa had a lower $\delta^{15}N$ than solo-seeded maize and maize interseeded with fescue, while in August the maize with alfalfa had $\delta^{15}N$ that was lower than maize interseeded with fescue. Differences in 112N fertilized maize were apparent only in August, with the maize interseeded with alfalfa having a lower $\delta^{15}N$ than the solo-seeded maize. The 224N fertilized maize in the three systems were never different.

Nitrogen fertilization was associated with differences in $\delta^{15}N$ signatures in several instances. In July the maize $\delta^{15}N$ in all three cropping systems was lower in the N fertilized treatments (112N and 224N) compared with the 0N rate for all three cropping systems. In August, the $\delta^{15}N$ signature in the 224N rate of solo-seeded maize was lower than the 0N and 112N rates, and the 224N rate of maize interseeded with tall fescue was lower than the 0N rate. Then in September, both N fertilized maize treatments were again lower than 0N for the solo-seeded maize and maize interseeded with fescue systems (Fig. 2). While the maize interseeded with alfalfa did not show a significant difference with N fertilization in August or September, we observed a trend for lower $\delta^{15}N$ in the fertilized treatments ($P < 0.2$). Additionally, the 224N fertilized solo-seeded maize and maize interseeded with fescue systems showed consistent differences by location (accounting for the significant treatment by location interaction in Table S2) where $\delta^{15}N$ was 0.9 to 1.4‰ greater at PDS than AARS. Similar to the maize, the interseeded fescue samples had lower $\delta^{15}N$ at the 224N rate compared with the 0N rate in July, but there was no difference in November. In contrast to both maize and fescue, the interseeded alfalfa samples at the 224N rate had higher $\delta^{15}N$ than alfalfa at the 0N or 112N rates in November, and while the same pattern was observed in July the difference between fertilizer rates was not significant (Fig. 1).

Nitrogen derived from atmosphere

Estimates of Ndfa showed high variability within treatments, and the low $\delta^{15}N$ values of the N fertilized reference crops resulted in negative Ndfa in several instances. Even so, interseeded maize Ndfa reached up to 43% while interseeded alfalfa reached up to 68%. Alfalfa and maize samples could be directly compared only at the July sampling date; the alfalfa at the 0N fertilizer rate had the greatest % Ndfa of ~ 55%, while the 112N and 224N rates of interseeded alfalfa as well as all three N rates of interseeded maize were not different with % Ndfa between − 51% and 27% (Tables 1 and 2). Interseeded alfalfa showed an increase in % Ndfa from July to November, and significantly greater % Ndfa for alfalfa at the 0N rate compared with alfalfa at the 112N and 224N rates. The solo-seeded alfalfa check had very similar Ndfa estimates to the unfertilized interseeded alfalfa in both July and November (54% and 64%, respectively). In contrast to the July samples, across the three maize sampling dates the maize interseeded with alfalfa did show significant treatment effects on Ndfa, with a lower % Ndfa observed for N fertilized interseeded maize when either the reference crop was either solo-seeded maize or maize interseeded with fescue (Table 2). Additionally, a significant trend with time was observed in interseeded maize Ndfa only when solo-seeded maize was used as the reference, where % Ndfa increased from July to September.

Two different reference crops were utilized for calculating % Ndfa estimates for both interseeded alfalfa and interseeded maize. Neither alfalfa nor maize % Ndfa estimates were statistically different between the reference crops ($p > 0.05$). For alfalfa, the fescue and solo-seeded maize reference crops could be compared in July at the 0N and 224N rates. That comparison revealed the Ndfa estimates generally differed by < 5% Ndfa due to the reference crop utilized, the one exception being 224N alfalfa at AARS with a difference of 14% Ndfa (Table 1). For maize interseeded with alfalfa the two reference crops (solo-seeded maize and maize with fescue) were compared at all three sampling times at the 0N and 224N rates, and as with alfalfa the Ndfa estimates were similar for the two reference crops (Table 2). Out of the 12

Table 1 Alfalfa % nitrogen derived from atmosphere (Ndfa) at two locations (AARS and PDS) and two sampling times (July and November)

Location	Interseeded alfalfa treatment	July		November
		% Ndfa (fescue)*	% Ndfa (solo maize)*	% Ndfa (fescue)*
AARS	Alfalfa 0N	55.5a	54.4a	67.7a
	Alfalfa 112N	–	−13.8b	–
	Alfalfa 224N	−37.4b	−51.8b	32.7b
	Solo alfalfa check	48.7	47.9	63.8
PDS	Alfalfa 0N	51.4a	55.1a	62.3a
	Alfalfa 112N	–	16.4b	–
	Alfalfa 224N	16.6b	20.8b	46.9b
	Solo alfalfa check	59.7	64.6	65.2

Two reference crops, fescue interseeded with maize and solo-seeded maize, were used for July calculations. Solo-seeded alfalfa check means are shown for comparison but were not included in statistical comparisons. Different lower-case letters within a column indicate differences in treatment means ($P<0.05$)

*Reference plant received same N management as alfalfa

**Negative values resulted when reference plant had lower $\delta^{15}N$ value than alfalfa

Table 2 Interseeded maize % nitrogen derived from atmosphere (Ndfa) at two locations (AARS and PDS) and three sampling times (July, August, and September)

Location	Treatment	July		August		September	
		% Ndfa (maize w/fescue)*	% Ndfa (solo maize)*	% Ndfa (maize w/fescue)*	% Ndfa (solo maize)*	% Ndfa (maize w/fescue)*	% Ndfa (solo maize)*
AARS	Maize + alfalfa 0N	8.1a	8.7a	11.9a	19.2ab	19.2a	9.5ab
	Maize + alfalfa 112N	–	−3.6ab	–	42.6a	–	21.3a
	Maize + alfalfa 224N	1.2b	−35.6b	−49.3b	−42.3c	−11.7b	−19.4b
PDS	Maize + alfalfa 0N	18.5a	8.8a	37.6a	11.6ab	36.6a	38.9a
	Maize + alfalfa 112N	–	10.7a	–	15.7ab	–	5.5ab
	Maize + alfalfa 224N	23.8b	27.3a	−8.0b	−4.3bc	14.4b	21.6a

Two reference crops, maize interseeded with fescue and solo-seeded maize, were used for calculations. Different lower-case letters within a column indicate differences in treatment means ($P<0.05$)

*Reference plant received same N management as interseeded maize

**Negative values resulted when reference plant had lower $\delta^{15}N$ value than interseeded maize

treatments comparisons of maize reference crops, 10 showed agreement within 10% Ndfa. The exceptions were interseeded maize at the 224N rate in July at AARS and the interseeded maize at the 0N rate in August at PDS, which differed by 37 and 26% Ndfa, respectively.

Biomass, N uptake, and Total Ndfa

Biomass and N uptake of maize at each sampling date and both locations responded to N application across all three cropping systems and was lowest at the 0N rate across all three cropping systems, but differences between the 112N and 224N rates varied by cropping system. For maize interseeded with alfalfa the 224N rate was greater in both biomass and N content, while for the solo-seeded maize biomass was similar but N uptake was greater in the 224N rate (Table 3). The yield advantage of the solo-seeded maize over the maize interseeded with alfalfa was 5.0–5.2 Mg ha^{-1} at the 0N rate and 0.8–3.5 Mg ha^{-1} at the 224N rate. The maize with interseeded tall

Table 3 Maize silage yield, total N content, and total Ndfa (N derived from atmosphere) in September at two locations (AARS and PDS)

Location	Crop system and N fertilization	Silage yield (Mg DM ha^{-1})	N content (kg N ha^{-1})	Ndfa (kg N ha^{-1})
AARS	Maize + alfalfa 0N	11.2d	82e	8.4a
	Maize + alfalfa 112N	18.9bc	158c	33.5a
	Maize + alfalfa 224N	20.4a	211b	−39.4b
	Maize + fescue 0N	16.9c	115de	–
	Maize + fescue 224N	20.8ab	219ab	–
	Solo maize 0N	16.4c	121cd	–
	Solo maize 112N	21.0a	195b	–
	Solo maize 224N	20.2a	226a	–
PDS	Maize + alfalfa 0N	9.6d	72e	27.3a
	Maize + alfalfa 112N	13.6bc	105c	4.4a
	Maize + alfalfa 224N	16.2a	152b	32.6a
	Maize + fescue 0N	12.9c	84de	–
	Maize + fescue 224N	18.2a	171ab	–
	Solo maize 0N	14.6c	100cd	–
	Solo maize 112N	17.0a	145b	–
	Solo maize 224N	19.6a	199a	–

The experiment included three maize cropping systems under three N fertilization regimes. Reference crop for Ndfa calculations was solo-seeded maize. Different lower case letters within a location in each column indicate differences in treatment means ($P < 0.05$). Results for July and August sampling dates are in tables S2 and S3

fescue had intermediate silage yield at both the 0N and 224N rates. These responses of maize biomass and N uptake to N rate and cropping system treatments were similar at the July and August sampling dates and were generally consistent at both locations (Tables S3 and S4). In contrast to the biomass and N uptake results, the response of total Ndfa of maize interseeded with alfalfa to N rate was limited, where only at AARS in September was total Ndfa lower at the 224N rate than the 0N and 112N rates. The Ndfa ranged from a negative estimate up to 24.8 kg N ha^{-1} and was not significantly different by N rate. There was not an overall significant trend with time in total Ndfa, although the 0N rate at PDS showed a trend for increasing total Ndfa over the course of the growing season (Fig. 3).

The alfalfa and tall fescue treatments had greater biomass and N content in July than in November and treatment differences varied by sampling date, while total Ndfa showed no difference between sampling dates (Fig. 3, Table 4). In July the 112N rate of interseeded alfalfa had greater N content than the 0N or 224N rates, but there was no difference between fertilization rates for biomass of interseeded alfalfa. Similarly, tall fescue biomass and N contents were not different at the 0N and 224N rates. However, in November we observed less biomass and N content in both 112N and 224N rates of fertilized alfalfa compared to the 0N rate, and less biomass for 224N fertilized fescue compared to 0N fescue. In addition, interseeded alfalfa had much greater biomass and total N content than interseeded fescue in both July and November. The total interseeded alfalfa Ndfa was affected by N fertilizer

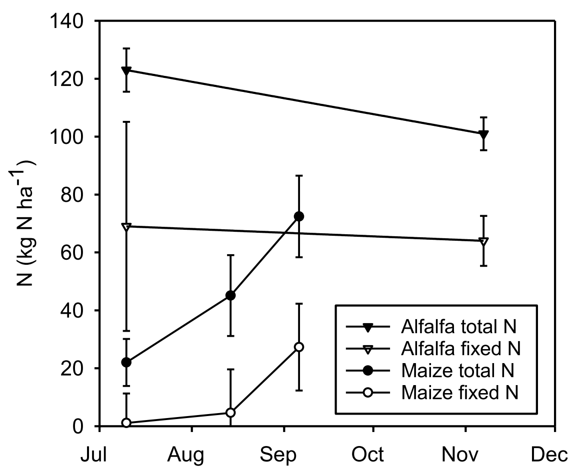

Fig. 3 Total aboveground N uptake and N derived from the atmosphere in interseeded maize and alfalfa at the PDS location at the 0N fertilization rate. Alfalfa N dynamics were not fully captured by the two sampling dates, as N content likely declined in August due to senescence then increased due regrowth following maize harvest in mid-September. Errors bars are standard error of the mean

Table 4 Biomass, total N content, and total Ndfa (N derived from atmosphere) for interseeded alfalfa and tall fescue at two locations (AARS and PDS) in July and November

Location	Crop and treatment	July			November		
		Biomass (Mg DM ha^{-1})	N content (kg N ha^{-1})	Ndfa (kg N ha^{-1})	Biomass (Mg DM ha^{-1})	N content (kg N ha^{-1})	Ndfa (kg N ha^{-1})
AARS	Alfalfa 0N	3.45b	113c	60a	2.47a	94ab	63a
	Alfalfa 112N	3.94a	152a	−22b	2.01b	77c	–
	Alfalfa 224N	3.63ab	134ab	−71b	2.10b	82bc	26b
	Fescue 0N	1.3c	41d	–	1.51c	29d	–
	Fescue 224N	0.8c	35d	–	0.88d	26d	–
PDS	Alfalfa 0N	3.64b	123bc	69a	2.76a	101a	64a
	Alfalfa 112N	4.19a	140ab	24b	2.43b	89abc	–
	Alfalfa 224N	3.72ab	125bc	26b	2.30b	84bc	39b
	Fescue 0N	1.45c	42d	–	1.95c	34d	–
	Fescue 224N	1.43c	56d	–	1.18d	28d	–

Reference crop for Ndfa calculations was solo-seeded maize for July and tall fescue for November. Different lower case letters within a column indicate differences in treatment means ($P < 0.05$)

rate, being greater in the 0N alfalfa compared with the 224N fertilized alfalfa when using interseeded tall fescue as the reference crop, and in July the Ndfa using solo-seeded maize as the reference crop was also greatest in 0N, intermediate in 112N, and lowest in 224N alfalfa.

Discussion

Interseeded alfalfa contributed substantial biological N_2 fixation and transfer to maize

Biological N_2 fixation by interseeded alfalfa was an important N source in several of the interseeded system treatments, as interseeded alfalfa contained up to 64 kg N ha^{-1} fixed from the atmosphere in July and November (depending on N fertilization rate). These estimates were lower than previous studies from Minnesota and Winnipeg that reported N fixation rates of 148 to 174 kg N ha^{-1} by unfertilized solo-seeded alfalfa in the seeding year under a regime of 1 or 3 harvests (Heichel et al. 1981; Kelner et al. 1997). The limited N_2 fixation by interseeded alfalfa was likely driven in part by competition from maize for light and water, which can reduce legume growth and lead to senescence of N_2-fixing nodules (Thilakarathna et al. 2016). Alternating solo-seeded strips of alfalfa and maize has been shown to stimulate alfalfa N_2 fixation through rhizosphere interactions, but the strips were spaced wide enough to allow light penetration to alfalfa (Wang et al. 2020). In contrast, in our study the maize rows were planted only 0.76-m apart, thereby increasing competitive interactions such as shading of the interseeded alfalfa. Alfalfa has been shown to shed nodules and fine roots and increase the mobilization of internal N stores when defoliated during harvest, suggesting N_2 fixation is temporarily impeded (Vance et al. 1979; Dubach and Russelle 1994; Schmitt et al. 2013). An analogous response likely occurred in this study in late summer due to senescence of interseeded alfalfa aboveground biomass. While alfalfa N_2 fixation appeared to resume along with aboveground re-growth following maize harvest, the shedding of nodules and heavy shading prior to maize harvest likely limited late summer N_2 fixation.

Nitrogen fertilizer reduced the importance of biological fixation to alfalfa N nutrition early in the growing season. The different locations showed different magnitudes of this effect; the negative estimates at AARS suggested near complete inhibition of N_2 fixation under the 112N and 224N rates, while PDS showed only a suppression of fixation. A similar response, where biological fixation was reduced by greater rates of N fertilization, has been previously observed in alfalfa (Eardley et al. 1985; Lamb et al. 1995). Alfalfa Ndfa estimates were consistently greater in November, where even the fertilized alfalfa derived 32–47% of N from biological fixation. Nitrogen fixation accounted for more alfalfa N in the post-July samples, likely due to more limited soil N supply during this period and continued maturation of alfalfa plants (Osterholz et al. 2021a). Nitrogen fixation by newly seeded alfalfa in monocultures and alfalfa/grass bicultures has been previously shown to increase dramatically over the course of the seeding year as plants develop (Heichel et al. 1984; Martensson and Ljunggren 1984; Bowman et al. 2002).

Transfer of biologically fixed N occurred from the interseeded alfalfa to the interseeded maize, particularly without N fertilization (Fig. 3). Our estimates were similar to N transfer estimates from unfertilized interseeded snail medic (*Medicago scutellata* L.) to maize of 0 to 63 kg N ha^{-1} (Smeltekop et al. 2002). Estimates of transfer of newly fixed N to maize were inconsistent in the fertilized interseeded maize, but we still observed a trend (albeit not statistically significant) of less Ndfa under fertilized than unfertilized interseeded maize. This result was consistent with a previous study that showed less transfer of N from alfalfa to maize under fertilized conditions (Shao et al. 2020). The estimates of maize Ndfa increased as the season progressed for the unfertilized maize (Fig. 3). In contrast, the fertilized maize did not show consistent trends as the growing season advanced, due to several negative estimates of Ndfa as well as the small contributions of N_2 fixation relative to the variability in the maize $\delta^{15}N$ signatures (i.e., small signal to noise ratio).

Nitrogen transfer from a legume to a neighboring non-legume can occur by several mechanisms (Fustec et al. 2009; Thilakarathna et al. 2016). In this experiment, we observed senescence of the interseeded alfalfa aboveground growth in August. This opened the possibility for maize to have taken up N rapidly released from the senesced alfalfa leaves, fine roots, and nodules under the relatively warm conditions of August and early September (Mohr et al. 1998; Johnson et al. 2012). However, we also observed evidence of biologically fixed N in the interseeded maize at the July sampling. As this sampling occurred prior to alfalfa senescence, it suggests there was direct transfer of N from alfalfa to maize which can occur through root exudates or mycorrhizal connections (Thilakarathna et al. 2016; Shao et al. 2020). Further research is required to elucidate the importance of specific N transfer mechanisms in interseeded maize/alfalfa.

Previous studies have shown competition for N is an important dynamic in interseeded legume/maize systems (Kurtz et al. 1952; Jellum and Kuo 1996). In this study, the lower yields of maize interseeded with alfalfa relative to both solo-seeded maize and maize interseeded with tall fescue clearly illustrated that alfalfa competed with maize for available soil N, despite the interseeded maize obtaining N fixed by the alfalfa. Soil N pools were measured in this experiment, and previously published results showed lower soil inorganic N in the interseeded maize/alfalfa system compared with solo-seeded maize at the 112N rate during the growing season and at all three N rates following maize harvest (Osterholz et al. 2021b). The competition for N led to a greater rate of N fertilizer needed to maximize yield of maize interseeded with alfalfa compared with solo-seeded maize (Osterholz et al. 2021a). Moreover, at the 0N rate the yields of maize interseeded with alfalfa were reduced by 33% compared with the solo-seeded maize and would likely not have been acceptable in a commercial production system. However, when 112 or 224 kg N ha^{-1} of N fertilizer was applied the interseeded maize yields were reduced by only 4 to 23% compared to the solo-seeded maize system. At these more realistic N rates, the proportion of N the maize obtained from alfalfa N fixation was smaller than at the 0N rate, but still accounted for up to 22% of maize N at PDS (where N fixation estimates were more reliable).

Competition for N between interseeded alfalfa and maize was clearly an important factor determining crop growth and yield, yet it must be noted that other factors also likely contribute to competitive dynamics between the crops. For instance, nearby plants can change the red/far red light ratio and thus influence maize growth patterns early in the growing season (Rajcan et al. 2004). Competition for nutrients other than N (e.g. P and K) as well as water could influence interseeded crop growth under limiting conditions (Kurtz et al. 1952; Morris and Garrity 1993; Zhao et al. 2019). Further research exploring the importance of these various factors will help elucidate the mechanisms underlying the performance of interseeded crops.

Maize and fescue reference crops produced consistent estimates of N_2 fixation and transfer

Solo-seeded maize, maize interseeded with tall fescue, and interseeded tall fescue all performed effectively as reference crops, highlighted by the similar $\delta^{15}N$ values of the three crops both with and without N fertilizer application. The primary advantage to including interseeded maize/tall fescue as reference crops was that it ensured a reference crop was available both during and after maize harvest. Additionally, as a perennial grass the tall fescue could feasibly be a useful reference crop for alfalfa N_2 fixation calculations in future growing seasons. The clear distinction of $\delta^{15}N$ signatures of the reference crops with and without N fertilizer reflects earlier research in mixed stands of legumes/grasses that indicated the necessity of applying a similar N fertilization strategy to the reference crop and the legume (Papastylianou and Danso 1989; Peoples et al. 2015).

N fertilization changed $\delta^{15}N$ signatures and N_2 fixation estimates

In this study maize fertilized with urea at moderate to high rates typically had lower $\delta^{15}N$ values than unfertilized maize, regardless of the presence of an interseeded crop. This result, along with the greater N uptake in fertilized maize, clearly showed urea was a major source of the N taken up by fertilized maize. A similar effect on interseeded tall fescue $\delta^{15}N$ was observed in July, but by November the fertilized and unfertilized fescue had similar $\delta^{15}N$ signatures, suggesting that by late fall soil N mineralization had replaced the contribution of urea to plant available N.

In several instances, particularly at the AARS location, the $\delta^{15}N$ values of the 224N fertilized solo-seeded maize and interseeded maize/tall fescue reference crops were lower than the recommended 2‰ threshold for natural abundance method recommended by Unkovich et al. (1994). The $\delta^{15}N$ signature of urea fertilizer is typically much lower than N from soil mineralization: for example, Bateman and Kelly (2007) found urea ranged from -0.8 to -5.9‰ whereas in this study the crops dependent on soil N mineralization (i.e., the 0N reference crops) averaged 4.5‰. A lower $\delta^{15}N$ of crops fertilized with urea has been observed in previous studies (Liu et al. 2017). As biologically fixed N has a similar or slightly higher $\delta^{15}N$ signature than urea (e.g., B value of -0.7), this made distinguishing between these sources and calculating Ndfa in the fertilized maize and alfalfa treatments challenging. At AARS the N required to reach optimal maize yield was close to the 112N rate meaning that the 224N rate supplied excessive N to the system (Osterholz et al. 2021b). The excess N and similarity between the signatures of urea and atmospheric sources likely contributed to the low $\delta^{15}N$ signatures of reference crops, thereby resulting in negative N fixation estimates in this treatment. This suggests that the natural abundance technique may not be effective for estimating N fixation when excessive rates of fertilizer with low N $\delta^{15}N$ signatures are applied. While most ammonia-based N fertilizers have delta signatures near zero to slightly negative, future studies on N fixation in N fertilized systems should consider utilizing an N source with a $\delta^{15}N$ signature more distinct from biologically fixed N to improve the ability to distinguish between biologically fixed N and fertilizer N. For example, nitrate-based synthetic fertilizers tend to have higher $\delta^{15}N$ signatures, and organic N sources are typically relatively enriched in ^{15}N (Freyer and Aly 1974; Bateman and Kelly 2007).

Conclusions

This study is the first to utilize ^{15}N natural abundance techniques in a field setting to determine N fixation by alfalfa interseeded with maize and demonstrate transfer to N from alfalfa to maize in the interseeded maize and alfalfa cropping system. Estimates of N fixation and N transfer were more challenging in N fertilized treatments due to smaller differences in $\delta^{15}N$ signatures between reference crops and interseeded alfalfa and maize; at one of the two locations the application of optimal and excessive N fertilization rates (for maize production) resulted in negative estimates of N fixation. Solo-seeded maize and tall fescue interseeded into maize were shown to be useful reference crops for alfalfa N_2 fixation, while solo-seeded maize and maize interseeded with fescue were appropriate reference crops for estimating N transfer to interseeded maize. Biological N_2 fixation by interseeded alfalfa in the seeding year contributed between 16 to 25 kg ha^{-1} of N to the interseeded maize/alfalfa system. Furthermore, maize accessed N fixed by interseeded alfalfa, both with and without N fertilization, which raises the potential to capitalize on N transfer processes to maintain efficient N cycling in the maize/alfalfa interseeding system. However, competition between interseeded crops remains an agronomic challenge as this was the dominant interaction observed. Future research on N dynamics of the interseeded maize-alfalfa system should explore management options to enhance N transfer from alfalfa to maize while minimizing the risk of N limitation of maize yields. Investigations of the specific N transfer processes as well as management approaches to minimize competition would help advance understanding and implementation of interseeded maize and legume cropping systems on farms.

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Declarations

Competing interests The authors declare no competing interests.

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