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Early interspecific dynamics, dry matter production and nitrogen use in Kernza (*Thinopyrum intermedium*) – alfalfa (*Medicago sativa* L.) mixed intercropping

Shoujiao Li[a,b], Ana Barreiro[b], Erik Steen Jensen[b], Yingjun Zhang[a] and Linda-Maria Dimitrova Mårtensson[b]

[a]College of Grassland Sciences and Technology, China Agricultural University, Beijing, People's Republic of China; [b]Department of Biosystems and Technology, Swedish University of Agricultural Sciences Alnarp, Sweden

ABSTRACT

The global interest in growing perennial grain crops such as intermediate wheatgrass (*Thinopyrum intermedium*) (Kernza) for production of food and feed is increasing. Intercropping Kernza with legumes may be a sustainable way of supplying nitrogen to soil and associated intercrop. We determined the competitive interactions between intercropped Kernza (K) and alfalfa (*Medicago sativa* L.) (A) under three inorganic nitrogen (N) rates N0, N1, N2 (0, 200, 400 kg ha^{-1}) and five species relative frequencies (SRF) (100%K:0%A, 75%K:25%A, 50%K:50%A, 25%K:75%A and 0%K:100%A) in mixed intercrops (MI) in a greenhouse pot experiment. After 11 weeks of growth. Kernza dry matter yield (DM) and N accumulated (NACC) were low, but alfalfa DM and NACC high at 0 kg N ha^{-1}. 200 and 400 kg N ha^{-1} fertiliser application increased the competitive ability (CA) of Kernza and reduced the CA of alfalfa. SRF had large impacts on alfalfa DM, NACC and NFIX only at 0 kg N ha^{-1} fertiliser, and insignificant impacts on Kernza at all N fertiliser levels, indicating that adjustment of SRF may not be an effective way to modulate the interspecific competition of Kernza. Further research on the other factors that influence the interspecific competition are warranted.

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Kernza; alfalfa; intercropping; interspecific interactions; competition; relative yield total

Introduction

Current global agriculture is dominated by the production of annual cereal crops, oilseeds and vegetables, with the use of tillage, fertilisation, irrigation and pesticides, which may lead to environmental problems including soil erosion, nutrient leaching and volatilisation, eutrophication of watercourses and overuse of water and energy (Smaje 2015). 'Perennialised' grain systems via breeding and planting new perennial grain crops have the potential to solve or reduce these problems (Dick 2016). Perennial crops have extensive rooting structures and permanent plant cover, which could facilitate water infiltration and improve water and nutrient utilisation. Perennial crops also can reduce soil erosion, increase soil organic matter, carbon sequestration, soil faunal diversity and improve wildlife habitat, while decreasing the requirement for tillage, labour, and fuel consumption (Pimentel et al. 2012).

The perennial Kernza crop was domesticated from forage intermediate wheatgrass (*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey) by The Land Institute, Salina, KS, USA (Culman et al. 2013). Kernza seed has high protein and fibre contents, which increase the interest in using it as food in restaurants, bakeries, brewers and commercial products for human consumption (Ryan et al. 2018). However, the grain yields of Kernza crop are currently much lower than annual wheat, and the seeds are about 20% of the size of most conventional wheat seeds. However, globally 1,450 M ha of cropland has been abandoned and 910 M ha are considered to be degraded or marginal for cultivation (Pimentel et al. 2012). Kernza may be grown on this degraded or marginal land and prevent it from being further serious degradation. Additionally, the Kernza straw can be harvested as biomass for biorefinery or grazed by livestock to provide additional income (Ryan et al. 2018).

Declines of the Kernza grain yield are often observed after two years of the perennial stand and may be linked to soil nutrient depletion (Tautges et al. 2018). Application of N fertiliser or non-chemical N management strategies, such as intercropping with legumes, may be needed to sustain reproduction of ageing perennial plants (Tautges et al. 2018). Previous research showed that the total biomass yield was largest in

CONTACT Linda-Maria Dimitrova Mårtensson linda.maria.martensson@slu.se Department of Biosystems and Technology, Swedish University of Agricultural Sciences, P. O. Box 103, 230 53 Alnarp, Sweden

alfalfa-intermediate wheatgrass intercrop compared to alfalfa-smooth bromegrass (*Bromus inermis* Leyss.), alfalfa-orchardgrass (*Dactylis glomerata* L.), intermediate wheatgrass-birdsfoot trefoil (*Lotus corniculatus* L.) and intermediate wheatgeass-kura clover (*Trifolium ambiguum* Bieb.) intercrops (Sleugh et al. 2000). Intercropping alfalfa with Kernza has proven to increase suppression of weeds through competition as compared to when Kernza was intercropped with yellow sweet clover (*Melilotus officinali* (L.) Lam.) or white clover (*Trifolium repens*) (Dick 2016).

Legume and cereal intercropping improves cereal grain yield and quality (Jensen 1996), yield stability (Raseduzzaman and Jensen 2017), the farmer gross margin (Bedoussac et al. 2015) and reduces weeds (Hauggaard-Nielsen et al. 2001), pest and diseases (Thorsted et al. 2006; Lopes et al. 2016) and nitrate leaching risk (Hauggaard-Nielsen et al. 2003) compared to sole crops. These advantages of intercropping occurred usually when species complementarity for nutrient and light resources is stronger than competition effects. The nutrient use efficiency like N use efficiency is higher in legume and cereal intercropping than sole crops, due to the N complementarity in time and space (Willey 1979), N transfer from legume to cereal (Thilakarathna et al. 2012) and alleviating inhibitory effect on symbiotic N_2 fixation (Li et al. 2009).

The total yield and the degree of resource complementarity attained are determined by interspecific and intraspecific competition between the intercrop components, which are influenced by the availability of resources and the relative frequency and density at which the component crops are sown (Hauggaard-Nielsen et al. 2006). Previous studies showed that under low N input conditions, the complementary acquisition of N from different sources would be dominant in intercropping systems (Jensen 1996; Yu et al. 2016), while the occurrence of N fertilisation would enhance the competitive ability of cereal and decrease the competitive ability of legume in intercropping (Stilmant et al. 2016). Interspecific competition could be modulated by N fertiliser application during plant critical growth stages in intercropping (Zhao et al. 2016; Xiao et al. 2018). Interspecific competition could also be determined by relative plant densities of the mixed species (Ofori and Stern 1987; Yu et al. 2016). Increasing relative plant density or frequency of one intercropped species increased its yield and competitive ability at the expense of the yield and performance of the companion species (Yu et al. 2016). Although the effects of N fertiliser and species relative frequency on species competition in intercropping have been reported, there is a lack of understanding to the interplay effects of N fertiliser and species relative frequency on the interspecific competition and yield in intercropping systems. For Kernza and alfalfa, recommended plant densities and N fertiliser levels when establishing sole crops of Kernza and alfalfa sole crops exists (Jungers et al. 2017; Berti and Samarappuli 2018). However, the optimum proportions of the species in mixed intercrops and the effects of N fertiliser level on establishment of a Kernza-alfalfa mixed intercropping system has not yet been determined.

The aim of this study was to determine the competitive interactions between intercropped Kernza and alfalfa during early growth and explore how N fertiliser and species relative frequency modulate the interspecific competitive or complementarity interactions to be able to optimise the establishment of the species in a mixed intercropping system. A greenhouse experiment was designed to determine the effects of the relative frequency of Kernza and alfalfa and the level of N fertilisation on the biomass yield, N use and interspecific dynamics. Sole crops and the mixed intercrops were grown under three inorganic N fertiliser levels and in five species relative frequencies to test three hypotheses: (i) the Kernza and alfalfa intercrop dry matter yields are higher as compared to the corresponding sole crops; (ii) without N fertilisation the Kernza is dominated by alfalfa due to the ability of alfalfa to fix N_2; and (iii) with increasing N fertiliser level the dominance of Kernza is expected to increase due to Kernza being more competitive than alfalfa for inorganic N.

Materials and methods

Experimental design

The experiment was conducted in a greenhouse at the Swedish University of Agricultural Sciences at Alnarp campus, Sweden (55°13′ N, 13°4′ E), from 18 January until 14 April 2018, under natural daylight conditions supplemented with 100 μmol m^{-2} s^{-1} photosynthetically active radiation for 16 h provided by high pressure sodium lamps. Day/night temperatures were 22°C/20°C and the relative humidity was kept at approximately 60%. The soil was collected from the top 20 cm at the SITES (Swedish Infrastructure for Ecosystem Science) Field Research Station Lönnstorp, Alnarp, Sweden. After collection in the field, the soil was sieved (4 mm), homogenised and (11 kg soil) placed in pots (diameter 285 mm, height 215 mm). The physical and chemical properties of the sandy loam soil were: total N 1.43 mg kg^{-1}, total phosphorus 0.36 g kg^{-1}, available phosphorus 51.0 mg kg^{-1}, total potassium 1.43 g kg^{-1}, available potassium 65.0 mg kg^{-1}, pH 7.26, and soil organic matter 9.00 g kg^{-1}.

The experimental design was comprised of two factors: (1) three inorganic N fertiliser levels N0, N1 and N2 (0, 200 and 400 kg N ha-1 or 0, 1.275 and 2.550 g N pot-1)(calculated using soil surface area per pot) and (2) five species relative frequencies (100 % K: 0 % A, 75% K: 25 % A, 50 % K: 50 % A, 25 % K: 75 % A and 0 % K: 100 % A). The high amount of N fertiliser used in this pot experiment was to secure a N fertiliser range wide enough to capture a reliable estimate of agronomical optimum nitrogen rate for Kernza biomass production. The plants grown in mixed intercropping and the total planting density after thinning was maintained at 16 plants per pot. The experimental design was a completely randomised design with five replicates. Kernza (*Thinopyrum intermedium* (Host) Barkworth & D.R. Dewey) seeds were taken from samples of seeds produced at the SITES Agroecological Field Experiment (SAFE) at Lönnstorp grown from seeds obtained from the Land Institute (Salina, KS) after the third round of selection. Alfalfa (*Medicago sativa* L.) seeds which pre-inoculated by *Rhizobium meliloti* were bought from the Swedish seed company Olssons frö AB.

Seeds of alfalfa and Kernza were sown on 18 January 2018. After two weeks, seedlings were thinned according to the five species relative frequencies. Soil moisture was monitored by a ThetaMeter type HH1 (Delta-T Devices) and 800 ml water was supplied every second day during the experiment. The N fertiliser treatment was equally split into three applications. The following amounts: 0, 1.214, and 2.429 g NH_4NO_3 (contained 0, 0.425, and 0.850 g N) were dissolved into 800 ml distilled water as three N fertiliser solutions and supplied to pots on day 15, 33 and 48 after sowing. No phosphates or potash fertilisers were applied in this experiment.

Sampling and measurements

The harvests were done on 5 April 2018 (day 77 after seeds sowing), all plant material was removed from the pots and separated into Kernza and alfalfa plants. First, alfalfa shoots were cut at the soil level, and then the entire root system of alfalfa and the entire shoot-root system of Kernza were gently separated from the soil. Last, the roots of Kernza were cut from the Kernza shoots. Kernza and alfalfa roots were washed in tap water. Kernza and alfalfa shoots and roots were oven-dried at 60°C for 72 h for the measurements of shoot and root dry matter. The dry matter of the species was expressed in g per plant.

Plant materials were grounded to a fine powder by using two milling machines (Retsch Cutting Mill SM200 (<2 mm) and Foss Cyclotec Mill 1093 (<1 mm)) for analyses of N concentrations (N %). The ^{15}N natural abundance method was used to estimate symbiotic N_2 fixation of alfalfa, hence 1–5 mg of each plant sample was placed in tin capsules and sent to the Section of Geography, Department of Geosciences and Natural Resource Management, Copenhagen University, Denmark, for isotope ratio mass spectrometer analysis of ^{15}N and N concentration.

The soil from pots was homogenised thoroughly and sieved by 2 mm sieve. A quarter of the soil was sampled and stored at −20°C for analyses of nitrate N and ammonium N concentrations. Mineral N from soil was extracted with 2 M KCl (Potassium chloride) solutions and analysed by continuous segmented flow analyzer (SEAL Analytical AA3). The concentration of soil mineral N was calculated by adding the concentration of soil nitrate N and ammonium N and presented as mg kg^{-1} soil.

Calculations

In this study, N accumulation (mg $plant^{-1}$) in each species was calculated as the product between N concentration and the shoot dry matter.

The ^{15}N natural abundance method was used to estimate symbiotic N_2 fixation of alfalfa. The natural abundance of the stable isotope ^{15}N in soil is often slightly higher than in the atmosphere. The ^{15}N abundance of N_2-fixing plant which using a combination of atmospheric N_2 and soil mineral N for growth is usually lower than that of non-N_2-fixing plants whose only source of N is from the soil. For this reason, differences in natural ^{15}N abundance between N_2-fixing plants and non-N_2-fixing plants can often be used to assess N_2 fixation (Shearer et al. 1983). Natural variation in ^{15}N abundance are expressed as $\delta^{15}N$ units, which are the parts per thousand deviation from atmospheric ^{15}N abundance. One $\delta^{15}N$ unit is 1% deviation from atmospheric $^{15}N/^{14}N$ ratio, and the $\delta^{15}N$ of atmospheric N_2 is set to 0. The $^{15}N/^{14}N$ ratio may be influenced by isotopic discrimination during N_2 fixation and in N transforming processes within the plant. To take such discrimination into account, the B value (the $\delta^{15}N$ of the legume grown in absence of combined N) is included when calculating Ndfa % (Carlsson and Huss-Danell 2003). The percentage of alfalfa total aboveground N accumulation derived from N_2 fixation (% Ndfa) was determined according to Equation (1), where $\delta^{15}N_{Kernza\ SC}$ means the $\delta^{15}N$ enrichment of Kernza sole crop being used as the non-fixing reference crop within each N fertiliser level (Shearer and Kohl 1986; Hauggaard-Nielsen et al. 2008). The B value is the $\delta^{15}N$ of alfalfa when grown in N free environment where atmospheric N is the only N source. The study from Unkovich et al. (2008) indicate that there is often a relatively small impact of cultivar or variety on shoot δ^{15}N for a particular legume species and that estimates of B can be similar in studies on the same legume undertaken in different laboratories. Therefore, A B value of −0.68 δ^{15}N units, which is the mean of the alfalfa B values from previous publications, was used in our study referring to research from Unkovich et al. (2008). The aboveground accumulation of N from the atmosphere was then used to calculate the amount of N_2 fixation (mg plant^{-1}) by alfalfa (Equation (2)).

Nitrogen balances (Equation (3)) for the cropping systems were determined to evaluate the net effect on the soil N pool when growing Kernza and alfalfa together as compared to the corresponding sole crops.

$$\%\ \mathrm{Ndfa} = \frac{\delta^{15}N_{\mathrm{Kernza\ SC}} - \delta^{15}N_{\mathrm{alfalfa}}}{\delta^{15}N_{\mathrm{Kernza\ SC}} - B} \times 100 \quad (1)$$

$$N_2\ \mathrm{fixation} = \mathrm{alfalfa\ \ dry\ matter} \times \frac{\mathrm{N\ concentration}}{100} \times \frac{\%\mathrm{Ndfa}}{100} \quad (2)$$

$$\mathrm{N\ balance} = \mathrm{fertilizer\ N} + N_2\ \mathrm{fixation} - \mathrm{N\ removal\ with\ shoot\ harvest} \quad (3)$$

The relative advantage of mixed intercropping compared with sole crops was evaluated by calculating the relative yield total (RYT; Equation (4)), which shows the relative amount of resources (light, water and nutrients) required under sole cropping to achieve the yield obtained in intercrop, indicating the efficiency of intercropping (or relative advantage) of using the resources of the environment compared with sole crops (De Wit and Van den Bergh 1965; Mead and Willey 1980; Dhima et al. 2007; Bitarafan et al. 2019). In the calculation of RYT, $Y_{\mathrm{Kernza\ IC}}$ and $Y_{\mathrm{alfalfa\ IC}}$ are the component yields of Kernza and alfalfa in mixed intercrops, and $Y_{\mathrm{Kernza\ SC}}$ and $Y_{\mathrm{alfalfa\ SC}}$ are the yields of Kernza and alfalfa sole crops. When the RYT is greater than one it indicates a greater advantage for mixed intercropping than for sole cropping. When the RYT is lower than one indicates less advantage for mixed intercropping than for sole cropping and equals to one indicates no advantages from mixed intercropping as compared to sole cropping.

$$\mathrm{RYT} = \frac{Y_{\mathrm{Kernza\ IC}}}{Y_{\mathrm{Kernza\ SC}}} + \frac{Y_{\mathrm{alfalfa\ IC}}}{Y_{\mathrm{alfalfa\ SC}}} \quad (4)$$

The advantage in N uptake by one species in intercropping over the other was determined by calculating the Competitive Ratio (Morris and Garrity 1993). The N Competitive Ratio of Kernza to alfalfa (CR_{KA}) in this study was calculated according to Equation (5), where $N_{\mathrm{Kernza\ IC}}$ and $N_{\mathrm{alfalfa\ IC}}$ are nutrient uptakes by Kernza and alfalfa mixed intercrops, $N_{\mathrm{Kernza\ SC}}$ and $N_{\mathrm{alfalfa\ SC}}$ are nutrient uptakes by Kernza and alfalfa sole crops, Z_{Kernza} is the sown proportion of Kernza in mixed intercropping and Z_{alfalfa} is the sown proportion of alfalfa in mixed intercropping. When CR_{KA} is greater than one, competitive ability in N taking up by Kernza is greater than alfalfa in mixed intercropping (Willey and Rao 1980; Li et al. 2001).

$$CR_{KA} = \frac{N_{\mathrm{Kernza\ IC}}}{N_{\mathrm{Kernza\ SC}} \times Z_{\mathrm{Kernza}}} \Big/ \frac{N_{\mathrm{alfalfa\ IC}}}{N_{\mathrm{alfalfa\ SC}} \times Z_{\mathrm{alfalfa}}} \quad (5)$$

The competitive relationship between Kernza and alfalfa was determined using the aggressivity value. Aggressivity is an index that is often used to indicate how much the relative yield increase in crop A is greater than that of crop B in an intercropping system (McGilchrist 1965; Dhima et al. 2007). The aggressivity of Kernza (A_K) in this study was calculated according to Equation (6). If A_K equals to zero, both crops are equally competitive; if A_K is positive then the Kernza is dominant; if A_k is negative then the alfalfa is dominant.

$$A_K = \frac{Y_{\mathrm{Kernza\ IC}}}{Y_{\mathrm{Kernza\ SC}} \times Z_{\mathrm{Kernza}}} - \frac{Y_{\mathrm{alfalfa\ IC}}}{Y_{\mathrm{alfalfa\ SC}} \times Z_{\mathrm{alfalfa}}} \quad (6)$$

Statistical analyses

All the measured variables fulfilled the assumption of normal distribution and the analysis of variance (ANOVA) was performed by general linear model, including Tukeý́s post hoc tests ($p < 0.05$) in Minitab 18.

Results

Biomass production

Kernza shoot dry matter was low (0.43–0.65 g plant^{-1}) at N0. The N fertiliser level N1 significantly ($p < 0.001$) increased the Kernza shoot dry matter compared to N0, but there was no difference between N1 and N2 (Table 1). Alfalfa shoot dry matter was high (2.57–10.0 g plant^{-1}) at N0. The effect of N fertiliser on alfalfa shoot dry matter was not significant, but the significant interaction between N and species relative frequency ($p < 0.001$) showed that alfalfa shoot dry matter yield was 2.1–3.9 times higher in the mixed intercrops with 75% Kernza than in other mixed intercrops and alfalfa sole crops at N0. Nitrogen fertilisation levels N1 and N2 reduced the alfalfa shoot dry matter yield in the mixed intercrops with 75% Kernza (Table 1).

Table 1. Shoot and root dry matter (DM) of Kernza and alfalfa under three N fertiliser rates (N) and five relative frequencies of Kernza (KRF). Data are presented as average ± standard error. F-statistics and significance from ANOVA are reported below the treatment means.

N	KRF	Shoot DM/g plant^{-1}		Root DM/g plant^{-1}	
		Kernza	alfalfa	Kernza	alfalfa
N0	100%	0.65 ± 0.03b	na	0.14 ± 0.02ef	na
	75%	0.45 ± 0.01b	10.0 ± 0.37a	0.23 ± 0.02cdef	3.35 ± 0.43a
	50%	0.43 ± 0.03b	4.74 ± 0.19bcd	0.12 ± 0.02f	1.86 ± 0.20bcd
	25%	0.61 ± 0.03b	3.26 ± 0.07ef	0.19 ± 0.03def	1.75 ± 0.05cd
	0%	na	2.57 ± 0.03f	na	1.29 ± 0.06d
N1	100%	3.53 ± 0.44a	na	0.41 ± 0.09abcdef	na
	75%	3.68 ± 0.22a	4.81 ± 0.62bc	0.66 ± 0.1abc	2.07 ± 0.20bcd
	50%	2.94 ± 0.30a	4.52 ± 0.31cde	0.84 ± 0.1a	2.74 ± 0.23ab
	25%	2.61 ± 0.49a	3.36 ± 0.21def	0.62 ± 0.12abcd	1.71 ± 0.20cd
	0%	na	3.50 ± 0.03cdef	na	1.63 ± 0.08cd
N2	100%	3.58 ± 0.23a	na	0.57 ± 0.11abcde	na
	75%	3.18 ± 0.15a	6.10 ± 0.45b	0.60 ± 0.05abcd	2.46 ± 0.21abc
	50%	3.70 ± 0.32a	3.59 ± 0.21cdef	0.75 ± 0.17ab	1.93 ± 0.06bcd
	25%	2.56 ± 0.41a	3.91 ± 0.14cdef	0.39 ± 0.08bcdef	2.05 ± 0.09bcd
	0%	na	3.55 ± 0.12cdef	na	1.57 ± 0.07cd
F-statistic					
Source of variation					
N		124.9***	16.4***	31.6***	0.1
KRF		3.12*	108.3***	2.95*	19.4***
N×KRF		1.86	27.9***	1.86	6.77***

Notes: Means within a column followed by different letters are significantly different according to Tukey's post hoc test ($p < 0.05$). na, no applicable, because there is no alfalfa in mixed intercropping which has 100% Kernza, vice versa.
*Significant at $P < 0.05$.
**Significant at $P < 0.01$.
***Significant at $P < 0.001$.

Kernza root dry matter was very low (0.12–0.23 g plant^{-1}) at N0. It was significantly increased ($p < 0.001$) by N1 in the mixed intercrops with 50% Kernza, but there was no difference between N1 and N2 (Table 1). Alfalfa root dry matter was high (1.29–3.35 g plant^{-1}) at N0. It was 1.8–2.6 times higher in the mixed intercrops with 75% Kernza compared to other mixed intercrops and alfalfa sole crops at N0, but N fertilisation level N1 and N2 reduced this alfalfa root dry matter in the mixed intercrops with 75% Kernza to the same level as alfalfa sole crops. There was no significant difference of alfalfa root dry matter between the mixed intercrops with 50% Kernza and alfalfa sole crops at N0, while alfalfa root dry matter was higher in the mixed intercrops with 50% Kernza than alfalfa sole crops at N1 (Table 1).

Nitrogen accumulation

Kernza shoot N accumulation was low (5.91–10.5 mg plant^{-1}) at N0. The effect of species relative frequency on Kernza shoot N accumulation was not significant ($p > 0.05$), but Kernza shoot N accumulation was increased by N fertilisation level N1 and with a further increase at N2 (Table 2). Alfalfa shoot N accumulation was high (91.1–288.1 mg plant^{-1}) at N0. It was 3.2 times higher in the mixed intercrops with 75% Kernza than alfalfa sole crops at N0. N fertilisation levels N1 and N2 reduced this alfalfa shoot N accumulation in the mixed intercrops with 75% Kernza, it was 1.6 times higher than alfalfa sole crops at N2 (Table 2).

Kernza root N accumulation was low (1.16–3.07 mg plant^{-1}) at N0. The N fertiliser level N1 significantly ($p < 0.001$) increased the Kernza root N accumulation compared to N0, but there was no difference between fertiliser levels N1 and N2 (Table 2). The alfalfa root N accumulation was high (23.8–52.6) at N0. It was 1.6–2.2 times higher in the mixed intercrops with 75% Kernza than alfalfa sole crops at N0, while N fertilisation levels N1 and N2 reduced it to the same level as alfalfa sole crops (Table 2).

Symbiotic N_2 fixation in alfalfa

The highest % Ndfa was observed in the mixed intercrops with 75% Kernza at N0 but no significant difference was detected between these samples and other mixed intercrops and alfalfa sole crops at N0 (Table 3). The N fertilisation level N1 did not change this trend but reduced the overall % Ndfa compared to N0. The % Ndfa in the mixed intercrops with 75% Kernza was further decreased by N2 compared to N0 and N1. The % Ndfa in the mixed intercrops with 50% Kernza was higher than alfalfa sole crop and the mixed intercrops with 75% Kernza at N2.

N_2 fixation was very high at N0. It was 3.4 and 1.7 times higher in the mixed intercrops with 75% Kernza and 50% Kernza compared to alfalfa sole crops at N0 (Table 3). The N fertilisation levels N1 and N2 reduced

Table 2. Shoot and root N accumulation of Kernza and alfalfa under three N fertiliser rates (N) and five relative frequencies of Kernza (KRF). Data are presented as average ± standard error. F-statistics and significance from ANOVA are reported below the treatment means.

N	KRF	Shoot N accumulation/mg plant^{-1}		Root N accumulation/mg plant^{-1}	
		Kernza	alfalfa	Kernza	alfalfa
N0	100%	6.99 ± 0.51d	na	1.71 ± 0.24cd	na
	75%	5.91 ± 0.37d	288.1 ± 22.2a	3.07 ± 0.34bcd	52.6 ± 7.61a
	50%	7.77 ± 0.66d	149.1 ± 3.34bc	1.16 ± 0.13d	31.0 ± 3.54bcd
	25%	10.5 ± 0.61d	112.9 ± 2.29cd	1.41 ± 0.33d	32.6 ± 0.85bcd
	0%	na	91.1 ± 1.87d	na	23.8 ± 1.18cd
N1	100%	59.2 ± 4.23c	na	7.67 ± 1.35abc	na
	75%	61.9 ± 2.18c	130.7 ± 22.1cd	10.6 ± 0.73a	24.7 ± 5.29cd
	50%	53.0 ± 4.14c	136.9 ± 6.01bcd	11.2 ± 0.95a	40.0 ± 2.13abc
	25%	55.1 ± 11.1c	100.1 ± 6.77cd	7.79 ± 1.76ab	22.2 ± 2.17d
	0%	na	105.2 ± 2.52cd	na	23.9 ± 1.84cd
N2	100%	98.3 ± 6.23ab	na	9.93 ± 1.97a	na
	75%	95.0 ± 4.22ab	187.5 ± 13.1b	10.4 ± 1.09a	46.0 ± 5.40ab
	50%	110.9 ± 7.60a	111.5 ± 8.19cd	11.6 ± 2.23a	32.7 ± 1.44bcd
	25%	75.4 ± 10.6bc	126.1 ± 4.37cd	6.13 ± 1.19abcd	35.8 ± 1.21abcd
	0%	na	114.2 ± 3.22cd	na	29.1 ± 0.94bcd
F-statistics					
Source of variation					
N		234.3***	15.9***	50.6***	6.61**
KRF		1.79	52.5***	3.85*	10.7***
N×KRF		2.72*	15.4***	1.29	5.86***

Notes: Means within a column followed by different letters are significantly different according to Tukey's post hoc test ($p < 0.05$). na, no applicable, because there is no alfalfa in mixed intercropping which has 100% Kernza, vice versa.
*Significant at $P < 0.05$.
**Significant at $P < 0.01$.
***Significant at $P < 0.001$.

this N_2 fixation in the mixed intercrops with 75% Kernza to the same level as alfalfa sole crops, and no significant difference between N1 and N2 was detected. Species relative frequency had no significant effect on N_2 fixation at N fertilisation levels N1 and N2.

Nitrogen balance and residual soil mineral N

The N balance was negative at N0, whereas N balance became positive at N1 and N2 (Table 3). The N balance was less positive in Kernza sole crops and in the mixed intercrops with 75% Kernza compared to alfalfa sole

Table 3. % Ndfa, N_2 fixation in alfalfa shoot, N balance and soil mineral N under three N fertiliser rates (N) and five relative frequencies of Kernza (KRF). Data are presented as average ± standard error. F-statistics and significance from ANOVA are reported below the treatment means.

N	KRF	% Ndfa	N_2 fixation/mg plant^{-1}	N balance	Soil mineral N/mg kg^{-1}
N0	100%	na	na	−0.11 ± 0.01h	2.73 ± 0.16d
	75%	95.0 ± 0.87a	274.2 ± 22.2a	−0.13 ± 0.00h	2.01 ± 0.08d
	50%	90.2 ± 0.98a	134.3 ± 2.02b	−0.18 ± 0.01h	2.39 ± 0.06d
	25%	92.6 ± 0.67a	104.6 ± 2.00bcd	−0.14 ± 0.01h	2.23 ± 0.06d
	0%	89.1 ± 1.00ab	81.3 ± 2.30cd	−0.16 ± 0.01h	2.19 ± 0.10d
N1	100%	na	na	0.33 ± 0.07g	2.96 ± 0.27d
	75%	78.5 ± 2.56cd	104.6 ± 20.1bcd	0.43 ± 0.02fg	2.29 ± 0.17d
	50%	79.5 ± 2.00bcd	108.6 ± 4.60bcd	0.63 ± 0.03ef	1.91 ± 0.05d
	25%	73.0 ± 0.87de	69.4 ± 3.48d	0.70 ± 0.02e	1.98 ± 0.06d
	0%	73.2 ± 1.77de	76.9 ± 1.20cd	0.82 ± 0.04de	1.97 ± 0.06d
N2	100%	na	na	0.98 ± 0.10cd	41.1 ± 3.00a
	75%	66.4 ± 4.45e	124.5 ± 12.8bc	1.16 ± 0.06c	31.0 ± 4.64b
	50%	85.5 ± 1.69abc	95.2 ± 6.34bcd	1.53 ± 0.05b	26.4 ± 2.37bc
	25%	79.1 ± 3.42bcd	99.6 ± 4.63bcd	1.93 ± 0.05a	18.2 ± 1.62c
	0%	70.4 ± 0.70de	80.3 ± 1.52cd	2.01 ± 0.03a	18.5 ± 4.60c
F-statistics					
Source of variation					
N		76.6***	39.2***	1952***	255***
KRF		6.64**	46.0***	75.1***	8.51***
N×KRF		6.98***	18.2***	31.8***	7.15***

Notes: Means within a column followed by different letters are significantly different according to Tukey's post hoc test ($p < 0.05$). na, no applicable, because there is no alfalfa in mixed intercropping which has 100% Kernza, vice versa.
*Significant at $P < 0.05$.
**Significant at $P < 0.01$.
***Significant at $P < 0.001$.

crop at N1. The N balance was less positive in Kernza sole crops, in the mixed intercrops with 75% Kernza and the mixed intercrops with 50% Kernza compared to alfalfa sole crops at N2.

The soil mineral N concentration was low (2.01–2.73 mg kg^{-1}) at N0. The N fertilisation level N1 did not increase soil mineral N concentration but N2 did increase ($p < 0.001$) soil mineral N compared to N0 (Table 3). Under the N fertilisation level N2, the soil mineral N was generally declining with increasing alfalfa relative frequency.

Interspecies interactions

The relative yield total was highest in the mixed intercrops with 75% Kernza, and it was tending to decrease with the decrease of Kernza relative frequency at all N fertiliser levels (Table 4). The relative yield total was high at N0 and reduced by N fertilisation, mainly by the first N fertiliser level N1 and not further with N2.

The N competitive ratio of Kernza to alfalfa was larger than one in the mixed intercrops with 25% Kernza and less than one in other mixed intercrops at N0 and N1 (Table 4). Under the N2 fertilisation level, the N competitive ratio of Kernza to alfalfa was larger than one in the mixed intercrops with 50% Kernza and less than one in other mixed intercrops. The N competitive ratio of Kernza to alfalfa was low at N0. Nitrogen fertilisation levels N1 and N2 increased the competitive ratio in the mixed intercrops with 75% Kernza, but no significant difference was observed between N1 and N2.

Table 4. Relative yield total (RYT), N competitive ratio (CR_{KA}) and Aggressivity of Kernza (A_K) under N fertiliser rates (N) and relative frequencies of Kernza (KRF). Data are presented as average ± standard error. F-statistics and significance from ANOVA are reported below the treatment means.

N	KRF	RYT	CR_{KA}	A_k
N0	75%	1.49 ± 0.03a	0.27 ± 0.02b	−3.22 ± 0.15d
	50%	1.25 ± 0.03b	0.69 ± 0.08ab	−1.18 ± 0.11c
	25%	1.18 ± 0.02bc	1.22 ± 0.09a	−0.34 ± 0.05ab
N1	75%	1.13 ± 0.01cd	0.99 ± 0.23a	−0.33 ± 0.24ab
	50%	1.06 ± 0.00de	0.70 ± 0.08ab	−0.46 ± 0.17abc
	25%	0.90 ± 0.02f	1.04 ± 0.24a	−0.22 ± 0.19ab
N2	75%	1.10 ± 0.02cde	0.61 ± 0.07ab	−0.83 ± 0.16bc
	50%	1.02 ± 0.02de	1.20 ± 0.16a	0.03 ± 0.15a
	25%	1.00 ± 0.04ef	0.71 ± 0.12ab	−0.38 ± 0.13ab
F-statistics				
Source of variation				
N		116.1***	1.33	58.9***
KRF		50.6***	5.32**	44.0***
N×KRF		6.59***	6.45**	25.5***

Note: Means within a column followed by different letters are significantly different according to Tukey's post hoc test ($p < 0.05$).
*Significant at $P < 0.05$.
**Significant at $P < 0.01$.
***Significant at $P < 0.001$.

Kernza aggressivity was negative at all treatments with the exception of the mixed intercrops with 50% Kernza at N2. Kernza aggressivity was increasing with the decrease of Kernza relative frequency at N0 (Table 4). The aggressivity was consistent throughout three Kernza relative frequencies at N1 and higher in the mixed intercrops with 50% Kernza than with 75% Kernza at N2. Kernza aggressivity was low at N0. The N fertilisation level N1 increased Kernza aggressivity in the mixed intercrops with 75% Kernza without further improvement at N2. The N fertilisation level N2 increased Kernza aggressivity in the mixed intercrops with 50% Kernza compared to N0.

Discussion

Our results confirmed the first hypothesis that the Kernza-alfalfa mixed intercrops had yield advantages as compared to sole crops. RYT is an index used to calculate the mixed intercropping benefits compared to sole cropping. The higher values of RYT (>1) in almost all combinations of Kernza and alfalfa, indicate the superiority of Kernza-alfalfa mixed intercropping over sole cropping in terms of the use of environmental resources for plant growth. The highest RYT existed in the mixed intercrops with 75% Kernza, mainly due to the contribution of yield advantages from alfalfa mixed intercrops, since the shoot and root DM and N accumulation of alfalfa in the mixed intercrops with 75% Kernza were much higher than in alfalfa sole crops meanwhile Kernza mixed intercrops kept the same yield and N accumulation as the Kernza sole crops. The alfalfa DM and N accumulation tended to decrease with the decrease of Kernza relative frequency at N0. It is possible that the intraspecific competition from alfalfa to alfalfa was stronger than the interspecific competition from Kernza to alfalfa, and alfalfa intraspecific competition was decreasing with the decrease of alfalfa relative frequency, thus one single alfalfa plant obtained more space, N nutrient and light resources in the mixed intercrops with high proportion of Kernza and low proportion of alfalfa than in alfalfa sole crops. Therefore, the alfalfa DM and N accumulation were highest in the intercrop with 75% Kernza at N0 which had the lowest alfalfa proportion. The values of RYT were lower at N1 and N2 than N0, which means that N fertilisation reduced the intercropping advantages of Kernza-alfalfa mixed intercrops. Numerous studies showed that the advantages of mixed intercrops are higher in low-N availability systems and organic farming (Willey 1979; Ofori and Stern 1987; Hauggaard-Nielsen et al. 2003; Bedoussac et al. 2015), because legumes can fulfil their N requirements by atmospheric N_2 fixation and save the soil available N for cereals, leading to a complementary use of N sources in low-input cropping systems (Bedoussac et al. 2015), legumes may also facilitate growth of associated cereals by transferring N (Rao et al. 1987), and cereals can stimulate N_2 fixation of legumes through competition for mineral N in the rhizosphere (Jensen 1996) or through root exudates (Li et al. 2016). However, high N fertilisation usually results in a reduction or inhibition of legumes nodulation and N_2 fixation (Peoples et al. 2002), reducing the complementary effect and intercropping advantages. Although the yield advantages were higher at N0 than N1 and N2, Kernza was the dominated species (A_K<0) and suffered from fierce competition (CR_{KA}<1) from alfalfa at N0. The balance of competition was important for the successful establishment of intercrops. The competitive ability of Kernza was improved by N1 and N2 fertiliser. Therefore, moderate fertiliser application N1 was recommended considering that the competitive ability of Kernza needs to be improved and the over aggressive growth of alfalfa needs to be suppressed.

Our results support the second hypothesis that without N fertilisation Kernza was dominated by alfalfa due to the ability of alfalfa to fix N_2. There are three types of plant interspecific interactions in cereal and legume intercropping systems: competition, facilitation and complementarity (Li et al. 1999; Bedoussac et al. 2015). Competition usually occur when one species modifies the environment to the detriment of another species, while facilitation occur when one species modifies the environment to the benefit of another species and complementarity occur when the intercropped species are not in competition for the same resources in time or space, or for a chemical form. In our study, competition and complementarity appeared in the Kernza-alfalfa mixed intercropping systems, since the DM and N accumulation of alfalfa intercrop yields were higher than alfalfa sole crops but the DM and N accumulation of Kernza mixed intercrops yields were similar with or lower than Kernza sole crops, indicating that for alfalfa mixed intercrops the complementarity effect was stronger than competition, while for Kernza the competition effect was stronger than complementarity. The asymmetric benefit for alfalfa mixed intercrops under unfertilised conditions was mainly due to the ability of alfalfa to fix N_2.

The results of % Ndfa showed that 90.2–95.0% of alfalfa shoot N accumulation was derived from atmosphere N_2. The amount of N_2 fixation by alfalfa shoot was even higher in alfalfa mixed intercrops than in sole crops at N0, because single alfalfa plant in the mixed intercrops occupied more space, light and other nutrient resources than in alfalfa sole crops due to the lower relative frequency in mixed intercropping. Furthermore, the amount of alfalfa shoot N fixed per g of shoot dry matter accumulated in all treatments was higher (25.0 kg N t^{-1}) than N fixed by subterranean clover which was less than 10.0 kg N t^{-1} when subterranean clover intercropped with Kernza in field (Hayes et al. 2017), and also higher than the previously established benchmark of alfalfa which was 18.7 kg N t^{-1} (Unkovich et al. 2010). The reason was probably that the environmental conditions in this greenhouse study were more advantageous to the growth of alfalfa as compared to complex field conditions, thus alfalfa had relative higher amount of N_2 fixation in our study, which resulted in high competitive ability and dominant of alfalfa in Kernza-alfalfa mixed intercropping systems, as indicated by the negative A_K, and the less than one CR_{KA} in most combinations.

Our results did not support the third hypothesis. With increasing N fertiliser level, the competitive ability for N and aggressivity of Kernza were increased, but did not consequently make Kernza become a dominant crop. The CR_{KA} and A_K of the mixed intercrops with 75% Kernza were higher at N1 and N2 than N0 means that N fertilisation increased the competitive ability of Kernza for N. The competitive ability of Kernza for N was even stronger than alfalfa in the mixed intercrops with 25% Kernza at N1and 50% Kernza at N2, however the A_K was less than or approximately equal to zero, indicating that factors other than N restricted the growth of Kernza and kept it dominated. We observed that alfalfa and Kernza were about the same height under fertilised conditions, which make the competition for light fierce due to the similar spatial niche for light. In our experiment, Kernza mixed intercrops looked smaller and thinner than Kernza sole crops, which suggest that the amount of light that was intercepted by Kernza mixed intercrops was less than that of sole crops. Reduced light availability restricts photosynthesis and the energy supply to Kernza roots resulting in reduced growth. Our results are in agreement with Hauggaard-Nielsen and Jensen (2001). They found that the indeterminate cv. *Salome pea* cultivar showed a much greater competitive ability towards barley probably due to increased competition for light, since the tall cv. *Salome pea* can grow into upper levels of the barley canopy potentially gaining increased access to higher incident light levels. Furthermore, the relative later sprouting time also affected the relative competitiveness and performance of intercropped Kernza. A meta-analysis showed that the negative effect of N on the relative yield of legumes was mitigated if the legume was sown before the cereal, contrarily, the positive effect of N fertiliser on cereals was reinforces if the cereal was sown before the legume (Yu et al. 2016). In our study, the germination and sprout of alfalfa was earlier than Kernza although they were sown at the same time, thus the negative effect of N fertiliser to alfalfa was mitigated, and resulted in a good performance of alfalfa mixed intercrops. Similar results of too aggressive alfalfa growth have been observed in alfalfa-Kernza intercropping in a field study by Dick et al. (2018). Berdahl et al. (2001) also found alfalfa dominated the intermediate wheatgrass-alfalfa intercrops in subsequent years from 1996 to 1998. Zhang et al. (2011) found alfalfa was the superior competitor when grown with corn and its productivity dominates the systematic biomass. Hu et al. (2017) reported that field pea had a relative greater competitive ability than maize for soil available N when intercropped together in strips. The reason could be that pea was sown earlier than maize which provided pea with competitive advantages over maize and offered the opportunity for the synergy of the interspecies with maize serving as a component crop (Hu et al. 2017).

Species relative frequency had larger impact on alfalfa than on Kernza, and N fertiliser was the other way around. Adjustment of species relative frequency may not be an effective way to modulate the interspecific interactions in Kernza-alfalfa mixed intercropping. N fertiliser can enhance the competitive ability of Kernza however cannot make Kernza became the dominant species in Kernza-alfalfa mixed intercrops. Other factors also need to be considered for establishing a competition balanced mixed intercropping system. Further research on the other factors, like light, that influences the interspecific competition in Kernza-alfalfa mixed intercrops are warranted.

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Notes on contributors

Shoujiao Li is a recently examined M.Sc. in Agricultural Science at the College of Grassland Sciences and Technology, China Agricultural University. Shoujiao specialised in perennial cereal grain crop model systems with focus on interspecies interference and N dynamics. The work presented in this article was conducted in the Cropping Systems Ecology Group at the Swedish University of Agricultural Science, under the supervision of Linda-Maria Dimitrova Mårtensson, Erik Steen Jensen and Yingjun Zhang.

Ana Barreiro is a Post Doc fellow in the Cropping Systems Ecology Group at the Swedish University of Agricultural Science, under the mentorship of Linda-Maria Dimitrova Mårtensson. Ana is specialised in soil microbial ecology, which includes symbiotic nitrogen-fixing bacteria in legume roots.

Erik Steen Jensen is a Professor in Agricultural Science and head of the Cropping Systems Ecology Group at the Swedish University of Agricultural Science. Erik has an well-documented experience in legume-cereal intercropping and sustainable agriculture. Erik has been co-supervisor of the work done by Shoujiao.

Yingjun Zhang is a Professor in Agricultural Science at the College of Grassland Sciences and Technology, China Agricultural University. Yingjun has long experience in grassland production systems including the use of legume intercropping.

Linda-Maria Dimitrova Mårtensson is an Associate Professor (Docent) in Agricultural Science in the Cropping Systems Ecology Group at the Swedish University of Agricultural Science. Linda-Maria has experience in plant ecology, soil microbial ecology, grassland ecology including grassland production systems. Linda-Maria has been the main supervisor of the work conducted by Shoujiao.

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