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Maintaining grain yields of the perennial cereal intermediate wheatgrass in monoculture *v.* bi-culture with alfalfa in the Upper Midwestern USA

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Abstract

Intermediate wheatgrass (*Thinopyrum intermedium*; IWG) is a perennial cereal crop undergoing development for grain production; however, grain yield declines of >75% are often observed after year 2 of the perennial stand and may be linked to soil nutrient depletion. Intercropping IWG with a perennial legume such as alfalfa (*Medicago sativa*) could benefit nutrient cycling while increasing agroecological diversity. Intermediate wheatgrass was established at five environmentally diverse sites in Minnesota, USA in (1) bi-culture with alfalfa, (2) non-fertilized monoculture and (3) monoculture fertilized annually in the spring with 80 kg N/ha. At northern sites where alfalfa growth was favoured, IWG grain yields were reduced in year 2 by growing IWG in bi-culture with alfalfa, relative to the monoculture systems. Across all sites IWG grain yield decreased by 90% in the non-fertilized monoculture, 80% in the fertilized monoculture and 65% in the bi-culture from year 2 to 4 and plant macronutrient concentrations decreased by 25–70%. In year 4, IWG grain yield was similar or greater in the bi-culture than the fertilized monoculture at three of the five sites and alfalfa biomass was correlated positively with grain yield, harvest index and nutrient uptake in the year 4 bi-culture. Chemical-nitrogen fertilization increased grain yields in year 2 but did not mitigate the decline in yields as stands aged. Intermediate wheatgrass in the bi-culture had similar yields and nutrient uptake and lower yield declines than the chemically fertilized stand at sites where alfalfa growth was maintained throughout the life of the stand.

Introduction

A large proportion of current global cropland was historically occupied by perennial polycultures of wild plant species; however, the agricultural revolution resulted in intensification of production practices, including tillage and annual crop production, which have focused largely on producing grain for human consumption and livestock feeding (Dewar, 2007; Cox *et al.*, 2010). Increasing land area used for annual row-crop agriculture has resulted in the pollution of marine and freshwater ecosystems, increased greenhouse gas emissions and declining biodiversity (Foley *et al.*, 2011). As an alternative to annuals, perennials have been proposed as grain crops (Glover and Reganold, 2010). Perennial crops have deeper rooting structures and a longer growing season than annual crops, enabling them to better capture precipitation and excess soil nutrients and prevent soil erosion (Glover *et al.*, 2010). Intermediate wheatgrass (*Thinopyrum intermedium* (Host) Buckworth & Dewey; IWG), a perennial grass first introduced into the USA as a forage crop, has been studied and selected for increased grain yield and cereal crop traits since 1988 (Wagoner, 1990). Presently, the grain yield potential of improved IWG lines remains significantly lower than for annual wheat; however, breeders predict that IWG grain yields could reach parity with annual wheat within 20 years if progress in yield improvement continues along its current trajectory (DeHaan *et al.*, 2014).

A re-thinking of the dominant production agriculture paradigm, in pivoting from annual to perennial crops, presents the opportunity to reintroduce polycultures across the landscape by establishing mixtures of perennial cereals and legumes (Wagoner, 1990; Dewar, 2007). Growing grasses and legumes in mixtures limits nitrogen (N) losses from cropping systems (Ledgard, 2001) and increases legume N_2 fixation (Suter *et al.*, 2015), grass protein content (Ta and Faris, 1987) and land use efficiency (Hauggaard-Nielsen *et al.*, 2008). Additionally, enhanced ecosystem services, such as reduced weed pressure (Tracy *et al.*, 2004), increased carbon (C) retention in soil (Sanderson *et al.*, 2013) and improved pollinator diversity through provision of habitat (Woodcock *et al.*, 2014), have been attributed to grass–legume forage

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mixes compared with grass monocultures. Furthermore, diversifying perennial mixes could contribute to greater food security and cropping system resilience through resource use complementarity (Howden *et al*., 2007).

As perennial cereals are a relatively new crop, few studies on perennial cereal–legume intercrops have been conducted. One of the first such studies was undertaken by Hayes *et al*. (2017) in south-eastern Australia, who examined the effect of seed placement of subterranean clover (*Trifolium subterraneum* L.) on clover establishment, persistence and N_2 fixation when planted in a mix with perennial wheat (*Thinopyrum* spp. × *Triticum aestivum* L.) breeding lines. Clover establishment and persistence was greatest when the two crops were sown in alternate rows and clover fixed sufficient quantities of N to support high perennial wheat grain yields. However, Hayes *et al*. (2017) did not observe effects of intercropping perennial wheat with legumes on grain yield and called for more research to investigate the link between legume resource access and companion cereal yields.

Declining soil nutrient availability, especially N, could also contribute to declining in seed production in IWG stands. Available soil N concentrations are generally low in grassland soils (Wedin and Tilman, 1990) and could become similarly low in perennial cereal systems as stands age and they more closely resemble grassland ecosystems. In addition to limiting the protein production necessary for seed development, N deficiency has been linked to decreased seed set (Hacker and Jones, 1971; Hebblethwaite and Ivins, 1978), which has been observed in ageing stands of IWG (Jungers *et al*., 2017). Therefore, as seed production is a high priority for maximizing productivity of perennial cereal stands, application of N fertilizer may be needed to sustain reproduction in ageing IWG plants. However, application of N fertilizer could reduce the sustainability of perennial grain production systems by increasing net energy inputs and nitrous oxide emission (Crutzen *et al*., 2008). Non-chemical N management strategies such as intercropping could increase IWG productivity while minimizing negative environmental impacts, as lower proportions of legume-derived N are generally lost from agroecosystems compared with chemical fertilizer N (Crews and Peoples, 2004). As N transfer from legumes to grasses has been observed in perennial grass–legume mixtures (Schipanski and Drinkwater, 2012), growing IWG for grain production mixed with a perennial legume could also improve the profitability of the system for growers by decreasing N fertilizer requirements over the life of the perennial cereal stand. Additionally, repeated removal of biomass from an agroecosystem without adequate nutrient replacement has been observed to lead to phosphorus (P) limitation (Aerts and Chapin, 1999). Growing legumes alongside IWG could reduce the need for chemical P inputs, as intercropping has been found to facilitate P uptake and utilization by a cereal crop (Li *et al*., 2003; Hinsinger *et al*., 2011).

Maintaining yield of perennial cereals such as IWG is a challenge that must be overcome to increase the adoption and impact of perennial cereal crops (Crews and DeHaan, 2015) and few studies have reported yields in second- and later-year perennial grain crops (Vico *et al*., 2016). Intercropping a perennial grain with a perennial legume could increase competitive interactions between intercrops, particularly below-ground, which could increase investment in reproduction by seed, thereby sustaining seed yields as stands age. Intercropping with a legume could also increase nutrient availability throughout the life of the stand through biological N input, soil P solubilization and stimulation of microbial activity and nutrient mineralization. The ability to maintain seed yields over the life of the stand is an important goal for improving the viability of perennial grains, as the environmental advantages of perennial grains could be limited by the frequent need for re-establishment.

To study the effect of intercropping IWG with alfalfa on grain productivity, a recent breeding line of improved IWG was established with the perennial legume alfalfa (*Medicago sativa* L.). Alfalfa was selected as a promising legume to intercrop with IWG because of its competitiveness, persistence, root biomass accumulation and 90–150% greater N_2 fixation than clover spp. when grown in a perennial grass–legume pasture mix (Peoples *et al*., 1998). The objective was to evaluate IWG grain yields, biomass yields and plant nutrient concentration and uptake in a bi-culture with alfalfa, compared with chemically fertilized and non-fertilized monocultures over years 2 through 4 of a perennial IWG stand.

Materials and methods

Experimental sites and design

The field experiment was conducted at five sites with differing soil types and climates across the state of Minnesota, USA (Table 1). The experiment was conducted on university research centres at all five sites, on ground that had been planted to conventional maize–soybean rotations in the 3 years prior to the study's establishment. Soil residual inorganic N levels ranged from 17 to 30 kg/ha, Olsen P levels from 0.009 to 0.033 g/kg and soil potassium (K) from 0.130 to 0.175 g/kg among sites at the time of intermediate wheatgrass establishment. Crookston (47° 48′N, 96° 36′W, 267 m a.s.l.) and Roseau (48° 50′N, 95° 51′W, 319 m a.s.l.) are located in northern Minnesota on a Wheatville loam (coarse-silty over clayey, mixed over smectitic, superactive, frigid Aeric Calciaquoll) and a Bearden silty clay loam (fine-silty, mixed, superactive, frigid Aeric Calciaquoll), respectively. Lamberton (44° 14′N, 95° 18′W, 348 m a.s.l.) and Morris (45° 38′N, 95° 54′W, 345 m a.s.l.) are located in southwestern Minnesota on a Normania clay loam (fine-loamy, mixed, superactive, mesic Aquic Hapludoll) and a Tara silt loam (fine-silty, mixed, superactive, frigid Aquic Pachic Hapludoll), respectively. Waseca (44° 04′N, 93° 31′W, 351 m a.s.l.) is located in southcentral Minnesota on a Webster clay loam (fine-loamy, mixed, superactive, mesic Typic Endoaquoll) (USDA-NRCS, 2018).

Field trials were established in a randomized, complete block design with four replicates, with 4.5 × 9 m plots. The Lamberton, Morris and Waseca sites were seeded in autumn 2011 and the Crookston and Roseau sites were seeded in spring 2012 due to high precipitation the previous autumn, which prevented planting. An improved population of IWG as of 2011, resulting from two cycles of mass selection at The Land Institute in Salina, KS (Zhang *et al*., 2016), was used to establish IWG stands. Cropping treatments were (1) bi-culture of IWG + alfalfa, (2) non-fertilized IWG monoculture and (3) chemically fertilized IWG. No N fertilizer was applied to the non-fertilized monoculture or the IWG + alfalfa bi-culture throughout the experiment. The fertilized monoculture received 80 kg N/ha in year 2 of the stand and 60 kg N/ha in years 3 and 4, which was applied in April of each year as urea. Given a history of repeated manure application at some of the sites, no P or K fertilizer was applied to IWG stands throughout the course of the study at any of the

Table 1. Seasonal mean temperature and total annual precipitation for five sites (Crookston, Lamberton, Morris, Roseau and Waseca) in Minnesota during 2013, 2014 and 2015

Site	Year	Season	Mean temperature (°C)	Total precipitation (mm)
Crookston	2013	January–March	−11.9	64
		April–July	13.0	228
		August–December	4.3	177
	2014	January–March	−15.1	32
		April–July	13.2	373
		August–December	5.0	128
	2015	January–March	−9.7	19
		April–July	14.3	302
		August–December	15.0	70
Lamberton	2013	January–March	−7.3	71
		April–July	14.3	332
		August–December	7.1	403
	2014	January–March	−9.8	112
		April–July	15.0	350
		August–December	7.6	77
	2015	January–March	−5.9	60
		April–July	16.1	395
		August–December	10.2	407
Morris	2013	January–March	−10.7	178
		April–July	13.1	402
		August–December	5.6	254
	2014	January–March	−12.2	73
		April–July	13.8	422
		August–December	6.1	261
	2015	January–March	−8.0	90
		April–July	15.5	364
		August–December	9.1	332
Roseau	2013	January–March	−11.8	134
		April–July	12.1	257
		August–December	2.8	327
	2014	January–March	−15.6	35
		April–July	12.4	304
		August–December	4.7	27
	2015	January–March	−11.1	45
		April–July	13.7	325
		August–December	7.2	65
Waseca	2013	January–March	−7.4	224
		April–July	14.6	625
		August–December	7.1	274
	2014	January–March	−11.4	232
		April–July	15.0	573
		August–December	7.3	256

(*Continued*)

Table 1. (*Continued.*)

Site	Year	Season	Mean temperature (°C)	Total precipitation (mm)
	2015	January–March	−6.8	137
		April–July	16.2	572
		August–December	10.7	551

sites. Weeds were managed with S-metolachlor, applied in April of each year.

Monocultures were planted at a rate of 18 kg/ha on 15 cm rows and in the bi-culture, IWG and alfalfa were planted in alternating 15 cm rows at 9 and 12 kg/ha, respectively. Alfalfa was seeded at the same time as IWG. For the current study, IWG and alfalfa were planted in alternating rows in the bi-culture following discussions with growers regarding their practices for establishing grass–legume bi-cultures with planting equipment considerations. Previous field trials examining IWG grain and biomass yields among row spacings of 15, 30, 45, 60 and 75 cm at establishment have shown that differences in row spacing in the year of establishment have no effect on yields in the following years of the stand, due to vegetative propagation of the perennial intermediate wheatgrass via tillering and rhizomal spread (J Jungers, unpublished data). Therefore, although row spacing differed among the monocultures and bi-culture at establishment, IWG growth compensation for different plant densities following the year of establishment enables comparisons of the effects of intra- and interspecific competition on IWG yield among cropping treatments. Alfalfa in the bi-culture was allowed to grow throughout the season and was not harvested until harvest of the IWG grain and harvest biomass, in mid-August.

Data collection

As IWG requires vernalization to induce flowering, grain production in the first year of the study (2012) was not comparable between the five sites due to southern sites (Lamberton, Morris and Waseca) being established in the autumn of 2011 and the northern sites being established in the spring of 2012, leading to incomplete flowering in northern IWG stands. Therefore, yield measurements were not conducted at all sites in first-year stands and first-year yields are not reported here. Data collection was initiated in 2013 at all sites in second-year IWG stands and continued in third- (2014) and fourth-year (2015) stands. The current study reports second-, third- and fourth-year yields for all five sites.

Grain yields were measured by cutting all IWG plants from two 0.5 m^2 quadrats per plot in mid-August, after hard dough and prior to grain shattering (corresponding to Zadoks growth stages 87–93; Zadoks *et al.*, 1974). Grain yields were measured at all environments except at Morris in 2014, where grain samples were lost due to consumption by rodents in storage. Grain was threshed from IWG spikes and mechanically de-hulled to separate grain from the lemma and palea. De-hulled grain was weighed to determine grain yield. Above-ground herbaceous biomass (stem + leaves) was harvested by cutting to a 10 cm stubble height, dried and weighed. Alfalfa biomass was separated from IWG biomass in the bi-culture treatment and alfalfa and IWG biomass from all cropping treatments was dried at 60 °C for 5 days before being weighed. Intermediate wheatgrass biomass (stem + leaves) and grain was analysed for macronutrients N, P and K concentration with near infrared spectroscopy (NIRS) (Shenk and Westerhaus, 1994) in 2013 (year 2) and 2015 (year 4) to examine the effect of synthetic N fertilization, an alfalfa intercrop and no fertility inputs, on IWG nutrient uptake over the life of the IWG stand. Results from NIRS were validated by the determination of N, P and K content in a sub-set of ground biomass samples using wet chemistry methods (Goering and Van Soest, 1975). Intermediate wheatgrass total biomass NPK concentration was calculated by adding the products of nutrient concentration and weight of the biomass and grain components. Nutrient export (NPK) was calculated for years 2 and 4 stands by multiplying nutrient concentration by IWG biomass and grain yields that were removed from the system.

Statistical analysis

Treatment effects on IWG grain and harvest biomass (stem + leaves, i.e. ‘straw’) yields, IWG harvest index and IWG whole-plant NPK concentration were determined with analysis of variance, conducted using the nlme package (Pinheiro *et al.*, 2017) in R version 3.2.2 (R Core Team, 2015). As repeated measures were undertaken on experimental units on an annual basis, repeated measures were specified with an auto-regressive covariance structure; however, as this model had an Akaike information criterion value greater than the model without a repeated-measures covariance structure, the more parsimonious model was used. Cropping treatment (non-fertilized monoculture, fertilized monoculture and IWG + alfalfa bi-culture) and stand age (year) were treated as factors and fixed effects and block was treated as a random effect. Site was treated as a fixed effect to examine the performance of the IWG monocultures and IWG + alfalfa bi-culture across precipitation and temperature gradients. Treatments were considered different if $P < 0.05$. Means separations were performed using the Tukey–Kramer honestly significant difference test at $\alpha = 0.05$. Pearson’s correlation coefficients were calculated for IWG grain yield, harvested biomass yield, alfalfa biomass yield, 3-year total alfalfa biomass production (calculated by summing alfalfa biomass from years 2, 3 and 4) and IWG plant NPK concentration using data pooled across all five sites. Pearson’s correlation coefficients were also calculated between years 2 and 4 NPK concentrations and years 2 and 4 NPK export, to evaluate whether nutrient removal earlier in the life of the stand negatively affected nutrient uptake in later years of the stand.

Results

Grain and harvest biomass yields

At Crookston in year 2 stands, IWG grain yields were similar between the monoculture treatments and lowest in the bi-culture (Fig. 1). At Lamberton, IWG grain yields were greatest in the fertilized monoculture, followed by the non-fertilized monoculture

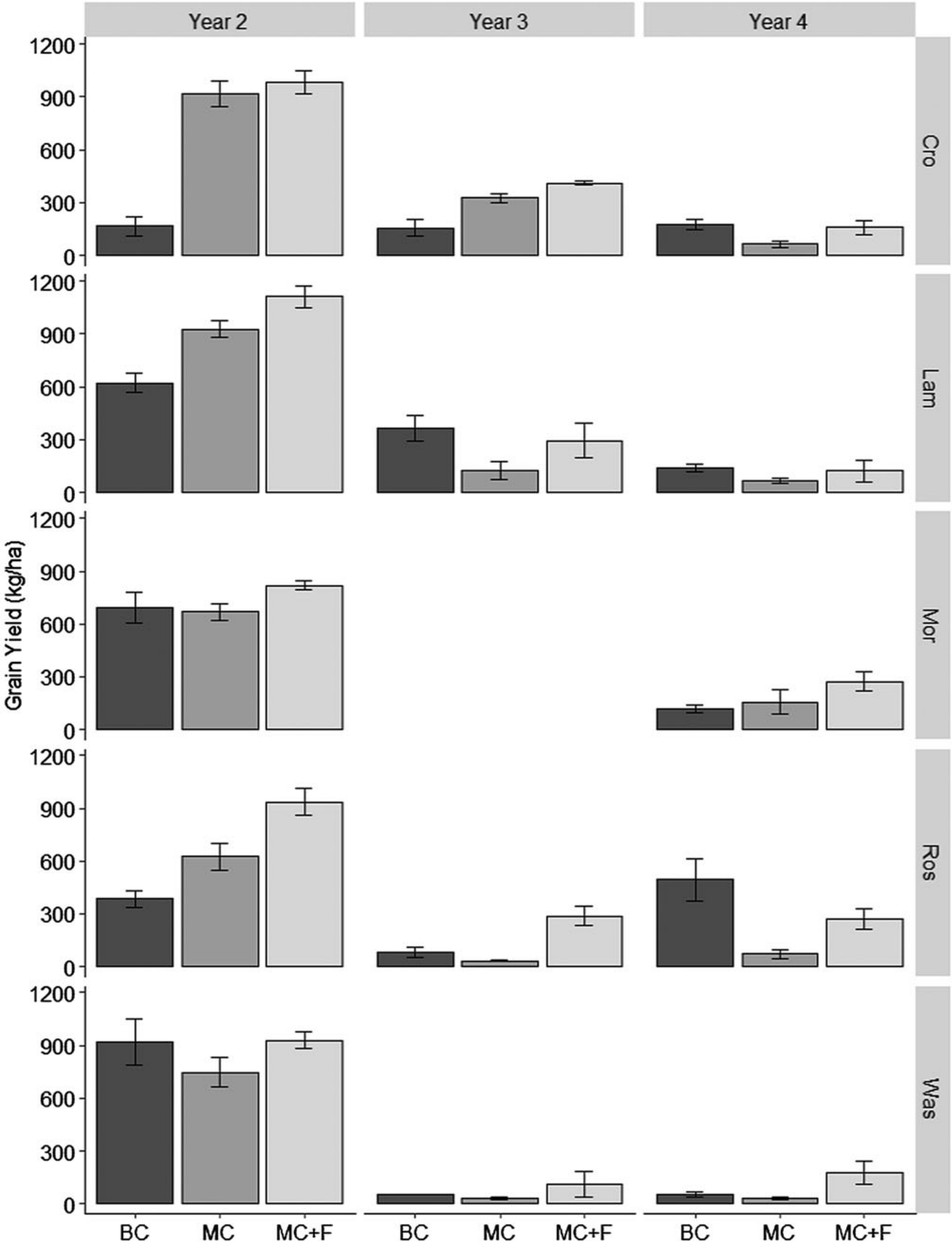

Fig. 1. Intermediate wheatgrass (IWG) grain yield in years 2, 3 and 4 of a non-fertilized monoculture (MC), fertilized monoculture (MC + F) and an IWG + alfalfa bi-culture (BC) at five sites across Minnesota from 2013 to 2015. No harvest grain samples were obtained from the Morris site in year 3. Error bars depict the standard error of the mean. Cro, Crookston; Lam, Lamberton; Mor, Morris; Ros, Roseau; Was, Waseca.

and were lowest in the bi-culture. At Morris, IWG grain yields were greater in the fertilized monoculture than the non-fertilized monoculture and the bi-culture, which had similar grain yields (Fig. 1). At Roseau, IWG grain yields were greatest in the fertilized monoculture, followed by the non-fertilized monoculture and IWG grain yields were lowest in the bi-culture. At Waseca, IWG grain yields were similar between the fertilized monoculture and bi-culture, which had greater yields than the non-fertilized monoculture (Fig. 1).

From year 2 to 3, averaged across all five sites, IWG grain yields declined by 80% in the non-fertilized monoculture, 71% in the fertilized monoculture and 63% in the bi-culture. In year 3 stands, IWG grain yields in the fertilized monoculture were generally greater than the non-fertilized monoculture and the bi-culture, with the exception of Lamberton, where IWG grain yields were similar between the fertilized monoculture and bi-culture and greater than the non-fertilized monoculture and at Waseca, where IWG grain yields were similar among all treatments (Fig. 1). From year 3 to 4, grain yields declined significantly in the non-fertilized and fertilized monocultures, but not in the bi-cultures at Crookston, Roseau and Waseca (Fig. 1). In year 4, IWG grain yields were similar between the fertilized monoculture and the bi-culture and greater than the non-fertilized monoculture at Crookston, Lamberton and Roseau (Fig. 1). In year 4 at Morris and Waseca, IWG grain yields were greater in the fertilized monoculture than the non-fertilized monoculture and bi-culture (Fig. 1).

Above-ground herbaceous IWG harvest biomass ('straw') yields in year 2 stands were greatest in the fertilized monoculture at Lamberton, Roseau and Waseca, similar between the non-fertilized and fertilized monoculture and greater than the bi-culture at Crookston, and similar among all cropping treatments at Morris (Fig. 2). In year 3, IWG herbaceous biomass yield was greatest in the fertilized monoculture at all sites except Morris, where yields were similar among all cropping treatments (Fig. 2). Herbaceous IWG biomass yields were similar between the non-fertilized monoculture and the bi-culture except at Crookston, where biomass yields were greater in the non-fertilized monoculture. Herbaceous biomass yields declined by 30% from year 2 to 4 as the IWG stand aged, but unlike with seed yields, there was no difference between years 3 and 4 biomass yields across treatments. In year 4, herbaceous IWG biomass yields were similar between the fertilized monoculture and the bi-culture and greater than the non-fertilized monoculture at Roseau. At Crookston, Lamberton, Morris and Waseca, biomass yields were greatest in the fertilized monoculture and were similar between the bi-culture and the non-fertilized monoculture (Fig. 2).

Although planted at the same rate at all sites, alfalfa biomass production in the bi-culture cropping treatment differed greatly among sites (Table 2). In the year following establishment (year 2), alfalfa biomass was greatest at the northern sites Crookston and Roseau, where it comprised 0.44 and 0.24 of total above-ground biomass, respectively (Table 2). At the southern sites Lamberton and Morris, alfalfa produced 320–333 kg/ha but only comprised 0.03–0.04 of total above-ground biomass and establishment at Waseca was poor (Table 2). Alfalfa biomass production in year 3 across all sites was low relative to years 2 and 4, probably due to the record cold winter experienced in the region from December 2013 to March 2014, which caused winterkill and decreased spring vigour (Table 1). Alfalfa biomass production trends among sites were similar in year 3, where despite the extreme cold temperatures, alfalfa was more successful at the northern sites, especially Crookston (Table 2), probably as a result of greater snow cover protection from winterkill than was present at the southern sites. Also, alfalfa growth was probably impacted negatively by higher-than-normal precipitation in spring at the southern sites (Table 1). While alfalfa survived the 2013–2014 winter and spring (as evidenced by an alfalfa stand being present in year 4), some winterkill and a slow start to growth in the spring of 2014 probably decreased alfalfa's ability to compete with IWG in intercropped stands, leading to low alfalfa biomass levels in year 3 stands. However, in year 4 when winter and spring conditions were warmer and more conducive to winter survival, alfalfa biomass production increased substantially from the previous years at the southern sites, especially at Morris and Waseca, and at the northern sites production levels were stable from year 3 to 4 (Table 2).

Harvest index in year 2 IWG stands was lower in the bi-culture (26–68 g/kg) than the non-fertilized and fertilized monoculture treatments (70–86 g/kg) at all sites except for Waseca, where harvest index was similar among all cropping treatments (81 g/kg; Fig. 3). Harvest index decreased by 67% on average across all sites and treatments from year 2 to 3 stands; it was similar among all cropping treatments in year 3 at Crookston, Roseau and Waseca and greater in the bi-culture than the monocultures at Lamberton (Fig. 3). In year 4, harvest index was greater in the bi-culture than the monoculture treatments at the northern sites Crookston and Roseau, similar among all treatments at Lamberton and Morris, and greater in the fertilized monoculture than the non-fertilized monoculture and the bi-culture at Waseca (Fig. 3).

Nutrient uptake

Intermediate wheatgrass plant N concentration in year 2 stands was greater in the fertilized monoculture than the non-fertilized monoculture and the bi-culture at Crookston, Lamberton and Waseca, and were similar among all cropping treatments at Morris and Roseau (Fig. 4). Across all sites and treatments, IWG plant N concentration was lower in year 4 than in year 2 by 40–46% (Fig. 4). In year 4, IWG plant N concentration was greatest in the fertilized monoculture, followed by the bi-culture and lowest in the non-fertilized monoculture, at Crookston, Lamberton and Waseca (Fig. 4). There were no differences in IWG plant N concentration among cropping treatments at Morris and Roseau in year 4 (Fig. 4).

Intermediate wheatgrass plant P concentration in year 2 stands was similar among cropping treatments at Crookston, Morris, Roseau and Waseca, and was greater in the bi-culture than the monocultures at Lamberton (Fig. 5). Intermediate wheatgrass plant P concentration was similar between years 2 and 4 stands at Crookston and Roseau and lower in year 4 than year 2 at Lamberton, Morris and Waseca by 26, 38 and 50%, respectively. Intermediate wheatgrass plant P concentrations in year 4 stands was greater in the fertilized monoculture and the bi-culture than the non-fertilized monoculture at Crookston, Lamberton and Waseca, similar among cropping treatments at Morris and greater in the bi-culture than the monocultures at Roseau (Fig. 5).

Intermediate wheatgrass plant K concentration in year 2 stands was greater in the fertilized monoculture than the non-fertilized monoculture and the bi-culture at Crookston and Waseca, greater in the fertilized monoculture and the bi-culture than the non-fertilized monoculture at Lamberton, and similar among all cropping treatments at Morris and Roseau (Fig. 6). Intermediate

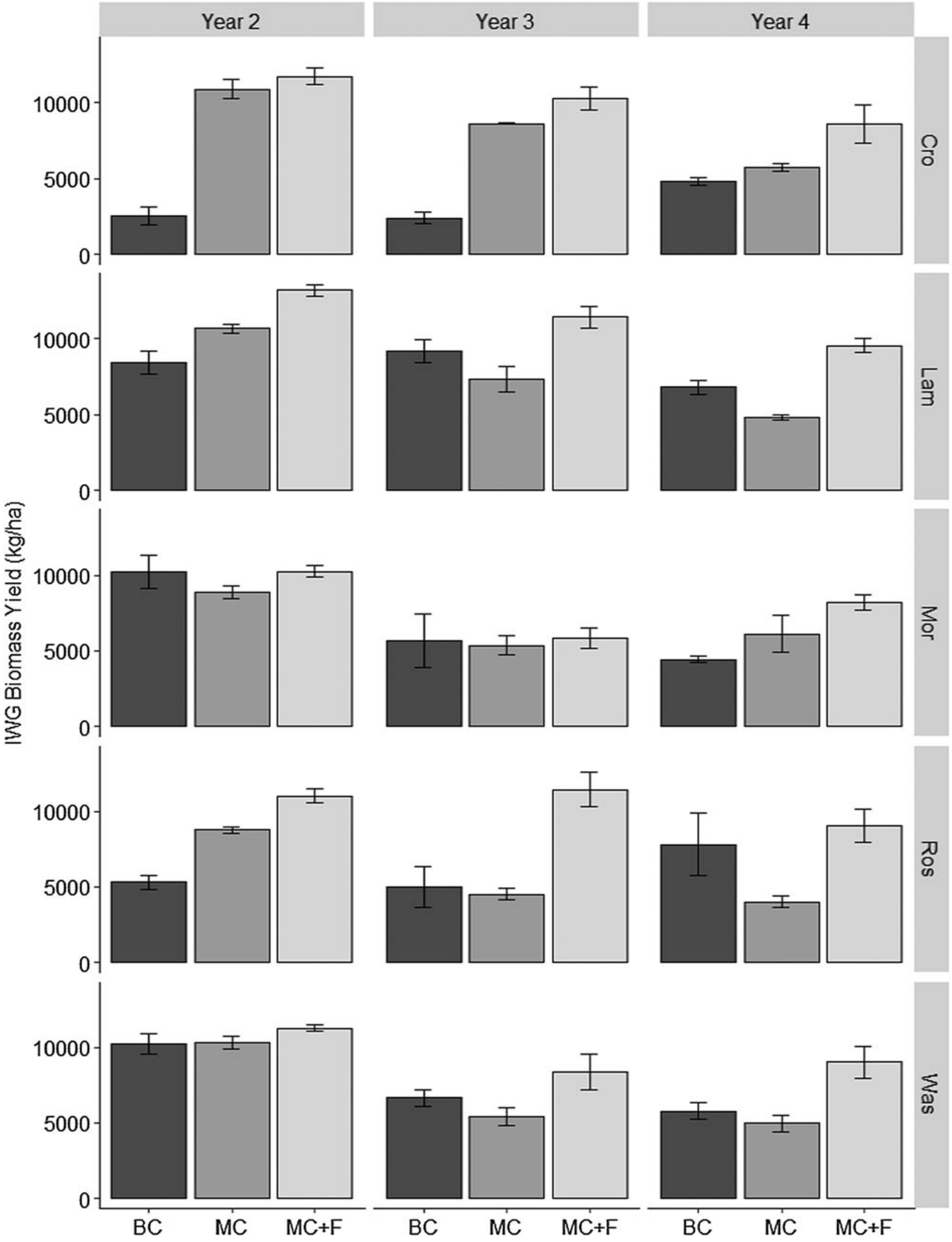

Fig. 2. Intermediate wheatgrass (IWG) herbaceous biomass yields at the time of grain harvest (mid-August) in years 2, 3 and 4 of a non-fertilized monoculture (MC), fertilized monoculture (MC + F) and an IWG + alfalfa bi-culture (BC) at five sites across Minnesota from 2013 to 2015. Error bars depict the standard error of the mean. Cro, Crookston; Lam, Lamberton; Mor, Morris; Ros, Roseau; Was, Waseca.

Table 2. Above-ground biomass production of the alfalfa component in years 2, 3 and 4 of an intermediate wheatgrass (IWG) + alfalfa bi-culture grown at five sites across Minnesota from 2013–2015

Site	Stand age (years)	Alfalfa biomass (kg/ha)	S.E.	Alfalfa: total biomass ratio	S.E.
Crookston	2	3985	925.5	0.6	0.11
	3	858	718.3	0.2	0.12
	4	1760	387.7	0.26	0.034
Lamberton	2	665	236.8	0.07	0.024
	3	60	20.7	0.01	0.001
	4	347	137.4	0.05	0.021
Morris	2	320	160.6	0.03	0.016
	3	23	30.4	0.02	0.001
	4	2446	592.0	0.34	0.065
Roseau	2	3310	263.9	0.39	0.039
	3	168	65.7	0.05	0.001
	4	390	292.7	0.09	0.074
Waseca	2	45	5.0	0.02	0.001
	3	9	9.4	0.02	0.001
	4	863	539.2	0.13	0.082

S.E., standard error of the mean.

wheatgrass plant K concentration was lower in year 4 stands than in year 2 stands by 42–70% and decreased from year 2 to 4 by a greater extent than did IWG plant N and P concentrations (Fig. 6). Intermediate wheatgrass plant K concentration in year 4 stands was greater in the bi-culture than the monocultures at the northern sites Crookston and Roseau, greater in the fertilized monoculture and the bi-culture than the non-fertilized monoculture at Lamberton, and similar among all cropping treatments at Morris and Waseca (Fig. 6).

In year 2, IWG grain yield was correlated negatively with alfalfa biomass production ($r = -0.82$; $P < 0.001$) across all sites (Table 3). Intermediate wheatgrass harvest biomass yield and harvest index was correlated negatively with alfalfa biomass production ($r = -0.59$; $P = 0.008$ and $r = -0.88$; $P < 0.001$, respectively). No correlations were observed between IWG grain yield, biomass yield and harvest index and IWG plant N and P concentrations in year 2, across all sites (Table 3).

In year 4, IWG grain yield was correlated positively with plant N ($r = 0.34$; $P = 0.013$), P ($r = 0.46$; $P < 0.001$) and K ($r = 0.38$; $P = 0.004$) concentrations (Table 3). Intermediate wheatgrass grain yield was not correlated with year 4 alfalfa biomass production but was correlated positively with 3-year total alfalfa biomass ($r = 0.28$; $P = 0.028$; Table 3). Correlations among IWG harvest biomass yield, plant nutrient concentrations and alfalfa biomass production were positive and similar to those of IWG grain yield (Table 3). In year 4, harvest index was correlated positively with IWG plant P ($r = 0.36$; $P = 0.011$) and K ($r = 0.35$; $P = 0.012$) concentrations, but not N. In year 4 stands, harvest index was not correlated with year 4 alfalfa biomass, but similar to grain yield, was correlated positively with 3-year total alfalfa biomass production (Table 3). Year 4 alfalfa biomass production was correlated positively with IWG plant N, P and K concentrations (Table 3).

Year 4 IWG plant N concentration was correlated positively with year 2 N export (via removal of harvest grain and biomass; $r = 0.37$; $P < 0.001$). Year 4 IWG plant P concentration was not correlated with year 2 IWG plant P concentration or P export, nor was year 4 IWG plant K concentration correlated with year 2 plant K concentration or K export. In year 2, a strong positive correlation was observed between IWG plant N and P concentrations ($r = 0.32$; $P = 0.016$), plant P and K concentrations ($r = 0.47$; $P < 0.001$) and plant N and K concentrations ($r = 0.72$; $P < 0.001$). In year 4, a strong positive correlation was observed between IWG plant N and P concentrations ($r = 0.74$; $P < 0.001$), plant P and K concentrations ($r = 0.84$; $P < 0.001$) and plant N and K concentrations ($r = 0.68$; $P < 0.001$).

Discussion

Intermediate wheatgrass grain yields in a second-year stand in the monoculture treatments ranged from 625 to 1110 kg/ha, similar to previously reported IWG yields in Minnesota (Jungers *et al.*, 2017). Lower yields were observed in the bi-culture, particularly at the northern sites Crookston and Roseau, where alfalfa biomass production was relatively high and comprised 240–440 g/kg of above-ground biomass. Competition from the alfalfa intercrop probably reduced IWG grain yields in the bi-cultures at the northern sites, as supported by the strong negative correlation between alfalfa biomass production and IWG grain and biomass yields and the lower yields in the bi-culture where alfalfa comprised a greater proportion of the stand. Alfalfa establishment may have been advantaged by lower spring rainfall and soil moisture levels in the northern sites, compared with the cooler, wetter conditions experienced during alfalfa establishment at the southern sites when seeded in the autumn of 2011. Spring establishment of alfalfa in IWG + alfalfa bi-cultures may favour

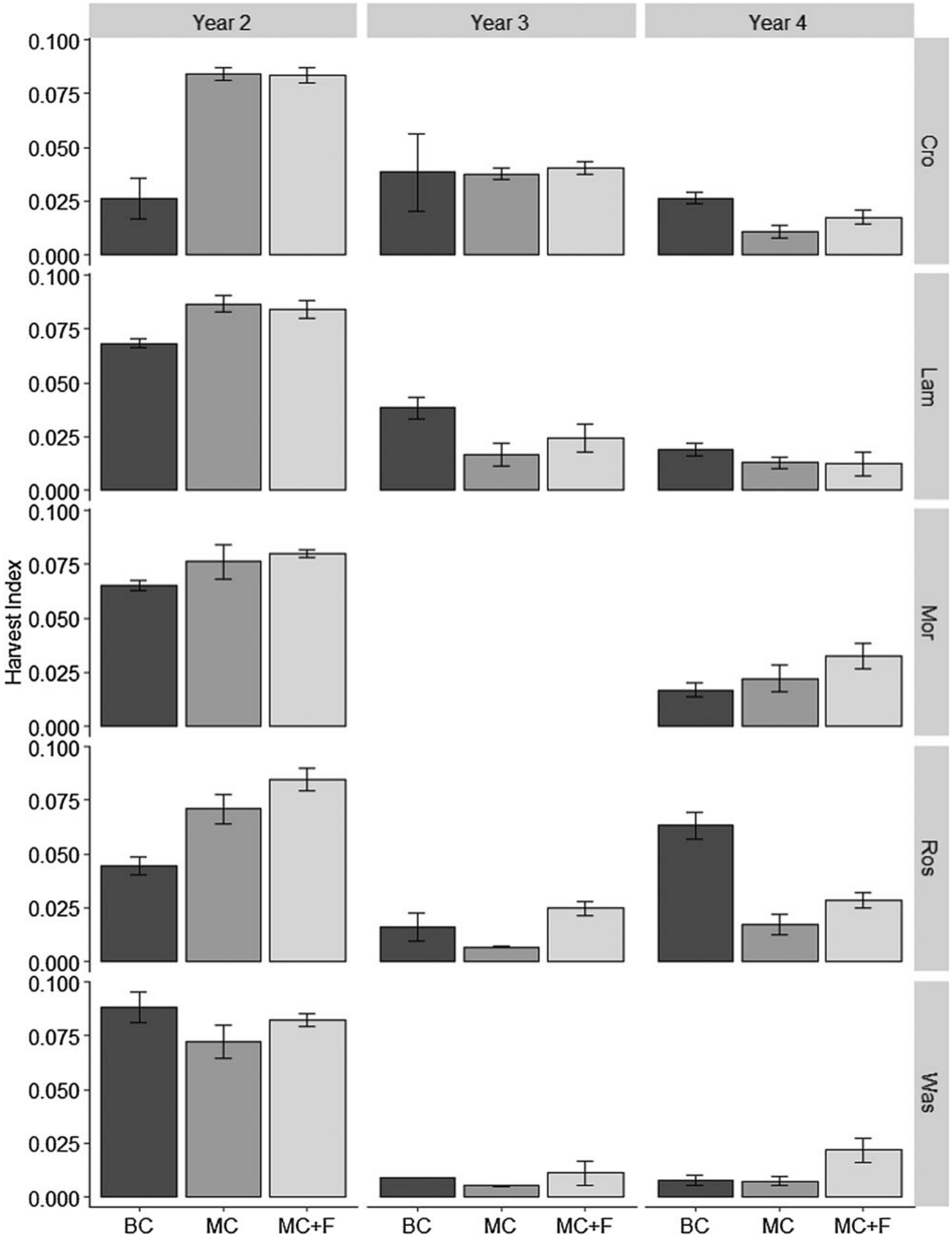

Fig. 3. Intermediate wheatgrass (IWG) harvest index in years 2, 3 and 4 of a non-fertilized monoculture (MC), fertilized monoculture (MC + F) and an IWG + alfalfa bi-culture (BC) at five sites across Minnesota from 2013 to 2015. Error bars depict the standard error of the mean. Cro, Crookston; Lam, Lamberton; Mor, Morris; Ros, Roseau; Was, Waseca.

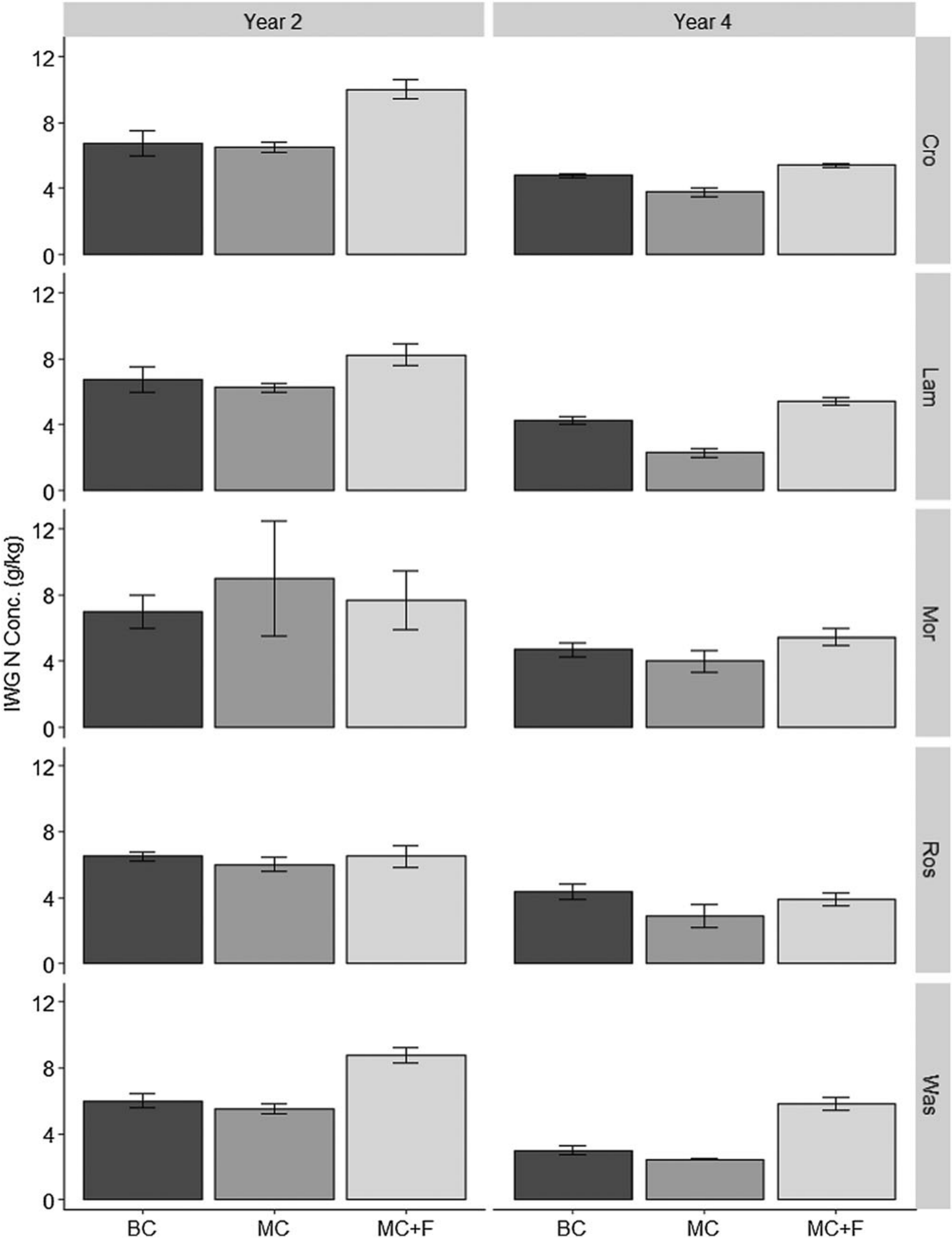

Fig. 4. Intermediate wheatgrass (IWG) above-ground plant nitrogen (N) concentration in years 2 and 4 of the perennial stand at five sites across Minnesota from 2013 to 2015. Error bars depict the standard error of the mean. Cro, Crookston; Lam, Lamberton; Mor, Morris; Ros, Roseau; Was, Waseca; MC, non-fertilized IWG monoculture; MC + F, fertilized IWG monoculture; BC = IWG + alfalfa, no N fertilizer applied.

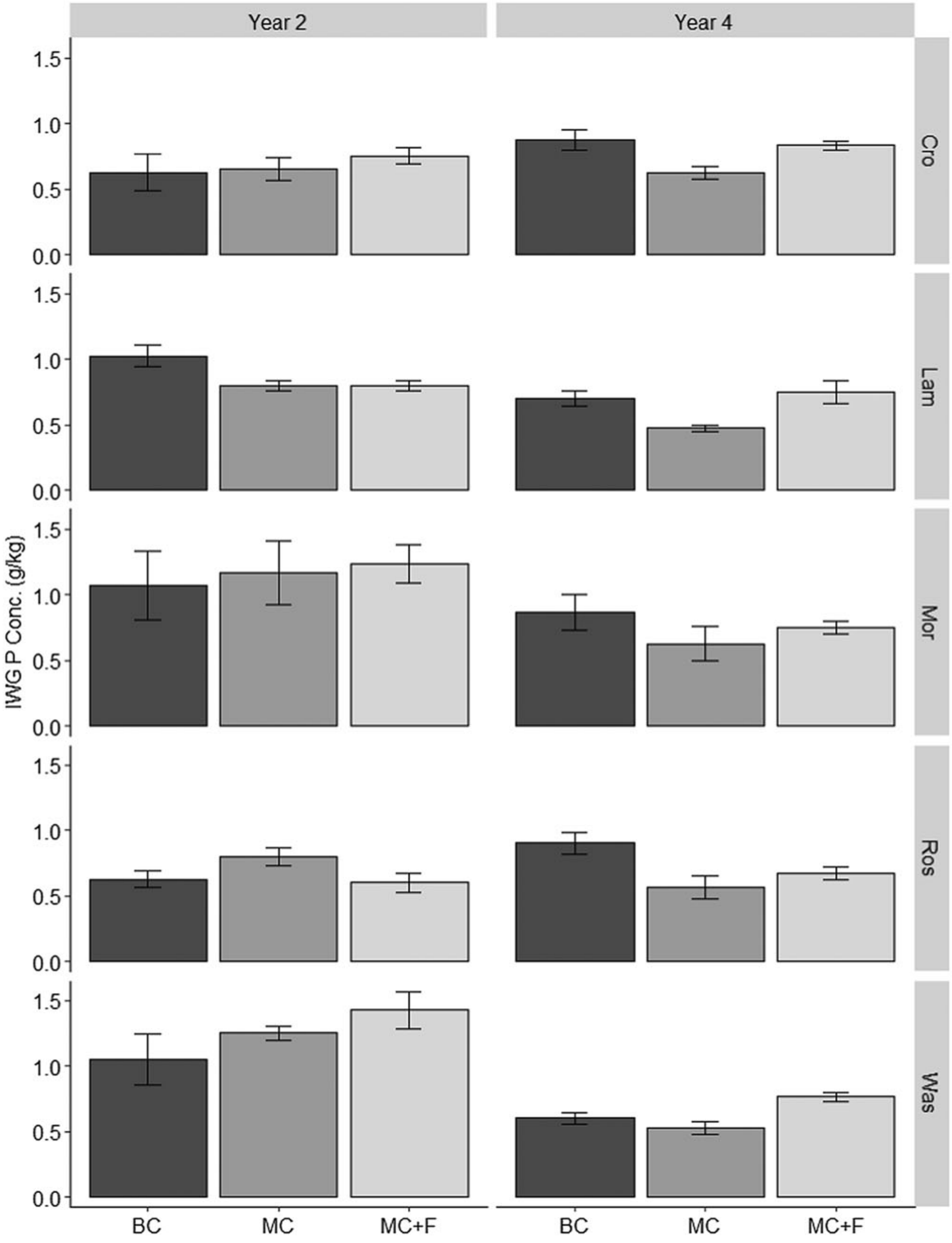

Fig. 5. Intermediate wheatgrass (IWG) above-ground plant phosphorus (P) concentration in years 2 and 4 of the perennial stand at five sites across Minnesota from 2013–2015. Error bars depict the standard error of the mean. Cro, Crookston; Lam, Lamberton; Mor, Morris; Ros, Roseau; Was, Waseca; MC, non-fertilized IWG monoculture; MC + F, fertilized IWG monoculture; BC = IWG + alfalfa, no N fertilizer applied.

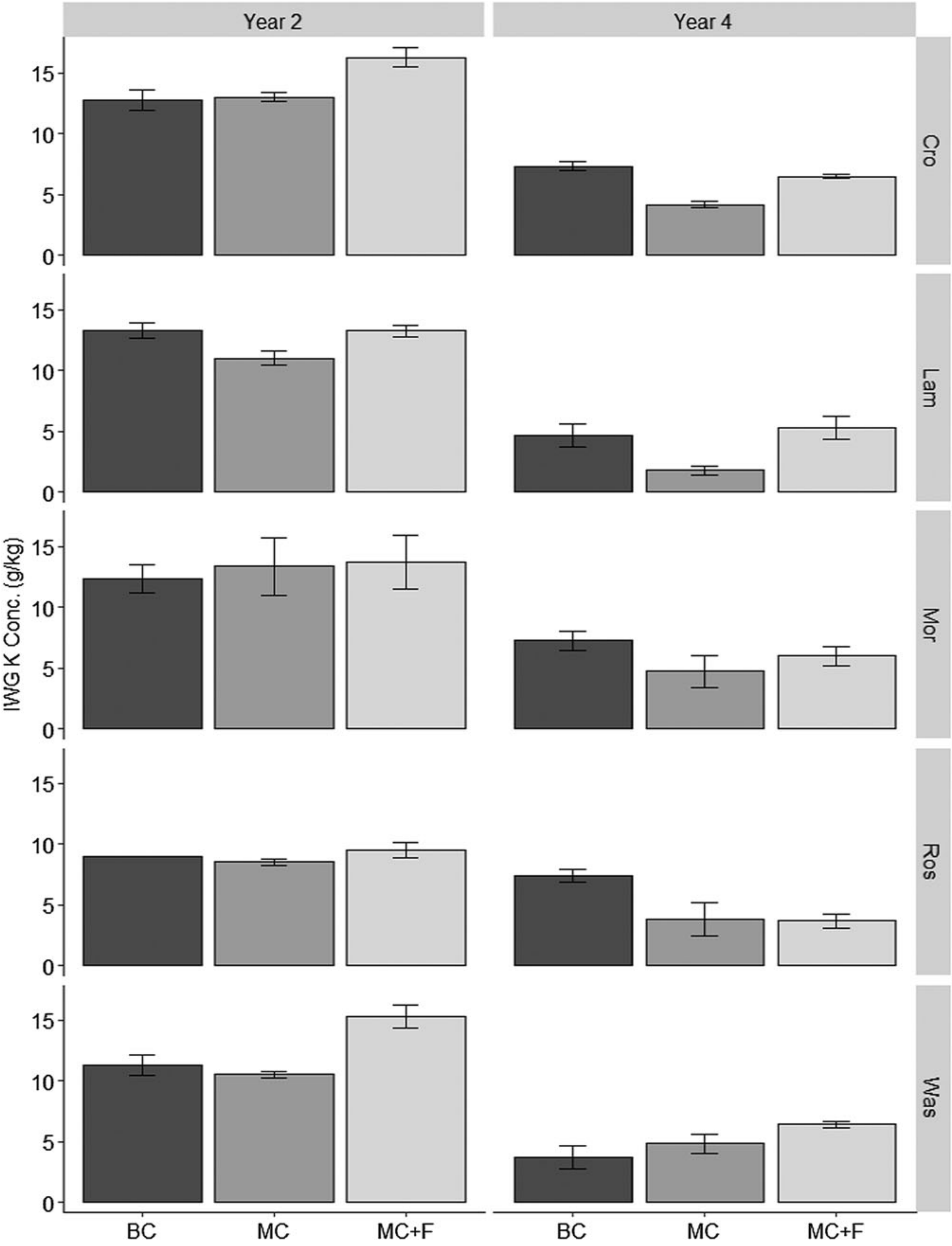

Fig. 6. Intermediate wheatgrass (IWG) above-ground plant potassium (K) concentration in years 2 and 4 of the perennial stand at five sites across Minnesota from 2013 to 2015. Error bars depict the standard error of the mean. Cro, Crookston; Lam, Lamberton; Mor, Morris; Ros, Roseau; Was, Waseca; MC, non-fertilized IWG monoculture; MC + F, fertilized IWG monoculture; BC = IWG + alfalfa, no N fertilizer applied.

Table 3. Pearson correlation coefficients (*r*) and significance tests (*P*, where *P* = 0.05)

	IWG plant N		IWG plant P		IWG plant K		Alfalfa biomass		3-Year cumulative alfalfa biomass	
	R	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Year 2										
IWG grain yield	0.20	0.133	0.07	0.591	0.23	0.090	−0.82	<0.001	–	–
IWG biomass yield	0.13	0.322	0.06	0.664	0.22	0.108	−0.59	<0.001	–	–
Harvest index	0.20	0.130	0.15	0.251	0.18	0.170	−0.88	<0.001	–	–
Alfalfa biomass	0.07	0.780	−0.44	0.070	−0.12	0.625	–	–	–	–
Year 4										
IWG grain yield	0.34	0.013	0.46	<0.001	0.38	0.004	−0.21	0.427	0.28	0.028
IWG total biomass yield	0.66	<0.001	0.55	<0.001	0.38	0.005	0.14	0.617	0.03	0.805
Harvest index	0.23	0.107	0.36	0.011	0.35	0.012	−0.22	0.416	0.36	0.007
Alfalfa biomass	0.55	0.033	0.64	0.010	0.59	0.022	1.00	–	0.51	0.041

Correlations between intermediate wheatgrass (IWG) grain and biomass yield in the bi-culture treatment and (1) IWG plant N concentration, (2) IWG plant P concentration and (3) IWG plant K concentration and (4) alfalfa biomass production, in year 2 (2013) and year 4 (2015) stands; averaged across five sites. In year 4, Pearson correlation coefficients were also calculated among all variables and 3-year total alfalfa biomass production (sum of alfalfa biomass in years 2, 3 and 4).

increased alfalfa density and biomass production in Minnesota, compared with autumn establishment. At southern sites where alfalfa biomass was lower and comprised <40 g/kg of the stand, such as Morris and Waseca, similar levels of biomass were observed among the monocultures and bi-culture in second-year stands. The exception among the southern sites was Lamberton, where while alfalfa comprised only 40 g/kg of above-ground biomass in the second-year bi-culture, IWG biomass and harvest index was lower in the bi-culture than in the monocultures in second-year stands. Whereas Morris and Lamberton had similar alfalfa biomass levels (320–330 kg/ha) and were the only two sites where a negative relationship was not observed between alfalfa biomass production and IWG grain yield, IWG biomass yields at Morris were similar among the second-year bi-culture and monocultures. Given similar or greater levels of macronutrient uptake in IWG in the bi-culture compared with the monocultures at Lamberton, the mechanism behind lower yields in the bi-culture at that site remains unclear. However, high levels of alfalfa biomass were achieved in the bi-culture in second-year stands; in this case, at the northern sites, increasing levels of alfalfa biomass were associated with lower yields in IWG when the two species were intercropped. Despite differences in IWG plant nutrient concentrations and yields among cropping treatments in year 2, the lack of correlation between these factors suggests that macronutrient uptake did not play a large role in yield differences among the cropping treatments in second-year stands, probably because of adequate soil residual fertility at the five sites. There was no relationship between alfalfa biomass production and IWG plant macronutrient (NPK) concentration in second-year stands, indicating that competition between alfalfa and IWG did not affect IWG nutrient uptake and that competition for water or light may have been more limiting for IWG grain production.

The steep decline in IWG grain yield observed from second- to third- and fourth-year perennial stands in the current study is similar in magnitude to that reported by others (Weik *et al.*, 2002; Jungers *et al.*, 2017). Few other studies have been published that report IWG grain yields beyond the second year of perennial stands and there is a need for more studies to examine IWG yields in third-year and older stands to identify the mechanisms of yield decline (Culman *et al.*, 2013). Some have suggested that an inherent trade-off exists between grain production and persistence in perennial plants, precluding productivity in later years of a stand (Vico *et al.*, 2016). In the current study, the significant decline in harvest index from second-year to third- and fourth-year stands suggests that IWG investment in flowering structures *v.* vegetative biomass may have declined as the stand aged. However, growing IWG alongside alfalfa increased IWG harvest index in fourth-year stands at the northern sites Crookston and Roseau compared with the monocultures, sites where alfalfa biomass production was high in earlier years of the stand, possibly as a result of better establishment from spring seeding. While year 4 harvest index was not related to same-year alfalfa biomass, the strong correlation between IWG harvest index and 3-year total alfalfa biomass production suggests that the effect of alfalfa on IWG resource allocation may be cumulative over the life of the stand and is possibly related to below-ground root interactions and/or facilitation of nutrient uptake of IWG by alfalfa. Weik *et al.* (2002) also observed improved grain yield persistence of IWG over the life of the stand when grown in a mix with undersown white clover (*Trifolium repens*) relative to a pure IWG stand and suggested that greater yields in the mixed IWG stand may have been due to greater N availability from leguminous N_2 fixation.

The >50% decline in IWG plant N concentration from year 2 to 4 in the non-fertilized monoculture suggests that endogenous N mineralization rates were not sufficient to support the N uptake requirements of IWG and that soil N depletion may be a factor in declining grain yields of perennial cereals as stands age. By comparison, the alfalfa intercrop increased IWG plant N concentration in year 4 after 4 years of intercropping alfalfa and IWG, relative to the non-fertilized IWG monoculture, suggesting increased N transfer or cycling from the alfalfa intercrop to IWG. Walley *et al.* (1996) also observed greater N concentration in bromegrass tissue after 3 years of intercropping with alfalfa, compared with monocropped bromegrass. In the same study, lower allocation of dry matter to bromegrass roots was observed when intercropped, compared with monocropped, which the authors attributed to enhanced soil N availability to bromegrass roots via N_2-fixing alfalfa (Walley *et al*., 1996). The majority of N taken up by non-N-fixing crops in mixes with legumes is derived from mineralization of legume residue, rather than direct N transfer from the roots (Peoples *et al*., 2009). As alfalfa is relatively efficient at soil N extraction, particularly from lower soil depths (Entz *et al*., 2001*a*), the alfalfa intercrop in the bi-culture may have enhanced soil N cycling and subsequent IWG access to soil N, apart from inputting N to the soil via biological fixation. Timing of N fertilization in the fertilized monoculture and N release in the bi-culture may also have impacted IWG grain yields in all years observed. While fertilizer application was chosen to occur in the spring to limit leaching losses of N, which are more likely to occur following autumn fertilizer application to grasses (Mangiafico and Guillard, 2006), some studies have found that autumn application of N fertilizer increases the number of flowering tillers the following year in cool-season grasses (Thompson and Clark, 1993; Loeppky and Coulman, 2002). More study is needed to compare spring *v.* autumn N fertilizer application effects on yield and nitrate leaching and to weigh trade-offs between productivity and ecological goals.

Beneficial effects of the bi-culture on IWG grain yields and N uptake were not observed until later years of the stand and were still lower than the N concentration of chemically fertilized IWG. Alfalfa grown in mixed swards with grasses has been found to transfer only 10–12% of fixed N to the grass intercrop in the first year, but this transfer can increase to 27–32% in the second and third years of the stand (Walley *et al*., 1996), which may explain why benefits of the alfalfa intercrop on IWG plant N concentration were not observed until year 4, relative to non-fertilized stands. While year 4 alfalfa biomass production was not correlated with IWG plant N concentration, 3-year total alfalfa biomass and grain yield and harvest index were correlated positively. Given these contrasting relationships, the N dynamics between IWG and alfalfa in the bi-culture remain unclear but suggest that alfalfa facilitation of IWG nutrient uptake may be indirect and cumulative over the course of several years.

Annual application of 80 kg N/ha to IWG monoculture mitigated the decline in grain yield with stand age and increased IWG biomass N in year 4, relative to the non-fertilized monoculture. Nonetheless, IWG biomass N levels in year 4 were still reduced in all cropping treatments, relative to year 2 of the stand, suggesting N limitation occurred over time regardless of N fertility management strategy. Increased applications of N fertilizer in later years of the stand may be needed to maintain N fertility and IWG N uptake, or other fertility management strategies may be needed to increase N cycling as IWG stands age. Soil nutrient depletion, which is common in wild perennial ecosystems (Vitousek and Howarth, 1991), could be a major factor contributing to grain yield and harvest index declines in perennial cereal stands. Previous rates of N fertilization suggested for IWG may not be adequately replacing the N removed in the grain and harvest biomass cumulatively over the life of the perennial stand, which was measured to be up to 140 kg N/ha in year 2 and 100 kg N/ha in year 3 of the current study.

Furthermore, a lack of understanding of P and K fertilization requirements in IWG grown for grain may be contributing to yield-limiting nutrient deficiencies and to a decreased ability of IWG to utilize available soil-N (Chapin, 1980). Decreased N uptake due to available P and K deficiency is supported by the correlations observed between IWG plant N, P and K concentrations in both years 2 and 4, which appeared to be more strongly related than year 4 plant N concentration and previous N export values. In the current study, no P and K fertilizers were applied in any treatment and greater declines in IWG plant K concentration were observed relative to N and P from a second- to a fourth-year stand. However, at the northern sites where alfalfa biomass production was greater, IWG plant K concentration was greater in the bi-culture than the monocultures and IWG plant P concentration was increased by N fertilization in four of five sites in fourth-year stands, was similar among the bi-culture and fertilized monoculture at three sites and was greatest in the bi-culture at Roseau in year 4. Intercropping a grain crop with a legume has been shown to increase grain yields when cereal crop and legume rhizospheres intermingle, through increased soil P availability from legume rhizosphere acidification (Li *et al*., 2007). Alternatively, the alfalfa intercrop could be increasing mycorrhizae inoculation and nutrient acquisition (Caldwell *et al*., 1985) or generally stimulating microbial activity, which has been observed to increase soil organic P mineralization under perennial plants (Chen *et al*., 2002; Crews and Brookes, 2014). Global P reserves are finite (Hinsinger *et al*., 2011) and P is often considered the most limiting nutrient in the long-term in organic systems (Entz *et al*., 2001*b*). Alternatives to increasing plant available P applying externally derived to cereal crops are needed to ensure sustainable grain production in the future.

Intermediate wheatgrass K uptake in monocultures *v.* a bi-culture with alfalfa was of interest because alfalfa has relatively high K requirements and responds strongly to K fertilization (Berg *et al*., 2005), suggesting that if K were to become a limiting nutrient in IWG stands over time, intercropping IWG with alfalfa could possibly exacerbate K depletion. Furthermore, K fertility management in perennial mixed stands may be further complicated by the closely related soil chemical properties of ammonium (NH_4^+) and K^+, which cause both ions to occupy the same fixation and exchange sites in soils (Liu *et al*., 1995). Intermediate wheatgrass plant K concentration, like N, declined in year 4 IWG relative to year 2 in all cropping treatments. However, IWG plant K concentration in the bi-culture was greater than the non-fertilized monoculture at four of the five sites and was greater than the fertilized monoculture at the northern sites where alfalfa produced significant levels of biomass throughout the 3 years. Alfalfa may have facilitated IWG biomass K uptake, supported by the positive relationship between alfalfa biomass production and IWG plant K concentration in year 4. Greater plant K concentrations in the fertilized monoculture and bi-culture in year 4 IWG may have been the result of NH_4^+ input, which released N from exchange sites, or from the stimulation of microbial activity and greater organic matter turnover, relative to the non-fertilized monoculture. Application of urea to the fertilized monoculture may have resulted in the release of K^+ from exchange or fixation sites in soils, as has been observed in other studies (Acquaye and MacLean, 1966; Bartlett and Simpson, 1967; Scherer, 1993), thereby increasing K availability.

The benefit to fourth-year harvest index and IWG plant nutrient concentrations gained from growing IWG with alfalfa observed at the northern sites and not at the southern sites is likely to be the result of better alfalfa establishment and growth in second- and third-year stands, which likely reduced IWG yields in the second year at Crookston and Roseau. In this case, where the yield potential of currently available improved lines of IWG remains greatest in the first and second years and decreases substantially in later years, the delayed benefit of intercropping IWG with a perennial legume may not be financially worth the wait. A better understanding of the mechanisms of yield decline in perennial cereal stands and improved IWG lines with greater yield potential as stands age, may lead to agronomic and genetic solutions to mitigate yield decline in the future. In this case, the benefits to harvest index and nutrient uptake of intercropping IWG with a perennial legume could result in meaningful increases in grain yield in older stands and prevent the costly termination and re-establishment of perennial crop stands.

More research is needed on agronomic methods to realize the benefits of growing IWG with legumes earlier in the life of the stand, such as establishing IWG in pre-existing alfalfa stands or establishing IWG with other legume spp. that establish faster and are less competitive for soil nutrients and moisture than alfalfa. Many of the proposed benefits of perennial cereal systems are associated with keeping perennial stands in production for several years; therefore, strategies are needed to maintain grain production as stands age. Yield potential of the crop is expected to increase from germplasm improvements via breeding; therefore, as IWG yield potential increases, growing IWG in a bi-culture with alfalfa or other legume spp. could be an economically viable alternative to external fertilizer inputs for moderating yield declines and maintaining grain yields as IWG stands age. More studies are needed to measure the effects of intercropping perennial cereals with perennial legumes in long-term stands (5 years or more) and to determine how soil fertility dynamics may differ in fertilized monoculture *v.* bi-culture stands. An understanding of these dynamics, particularly as stands age, would enable optimization of fertilizer application timing (e.g. autumn *v.* spring *v.* split applications) or legume intercrop management to time nutrient release to correspond to periods of growth where nutrient uptake correlates with grain production.

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