Agricultural and biofuel implications of a species diversity experiment with native perennial grassland plants

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A B S T R A C T

Two primary approaches to perennial biofuel crop production studied so far are fertilized grass monocultures and low-input high-diversity grasslands. While high-yielding perennial grass varieties are being developed in fertilized monocultures, breeding for yield in low-input high-diversity systems would be difficult. Before initiating breeding for low-input systems, it is therefore important to know the minimum number of functional groups and species required for maximum biomass harvest from low-input grasslands. We controlled the number of perennial grassland species in 168 plots in Minnesota, USA. Species were selected at random from a pool of 18, and 1, 2, 4, 8, or 16 were planted in each plot. Aboveground biomass was measured annually, and the plots were burned each spring. We found a strongly positive log-linear relationship between average annual aboveground biomass and planted species number, but a large proportion of plot-to-plot variability remained unexplained. We performed a conditional analysis of the aboveground biomass data to determine whether considering species identity would reduce the minimum number of species necessary in order to achieve yields similar to the highest diversity treatments. A model that accounted for the presence of legumes in general, and for the presence of the legume species *Lupinus perennis* in particular, showed no increase in biomass yield with increased species number. Over 11 years, average yields of *L. perennis*/C_4 grass bicultures were similar to those of 16-species (maximum diversity) plots, and both were >200% greater than the average of monocultures. Thus, under low-input conditions, the choice of the appropriate few perennial plant species for each location might result in systems with biomass yields similar to those from high-diversity systems. Because breeding biofuel crops in diverse mixtures would introduce complexity that is unwarranted in terms of maximum biomass yield, the first biofuel crop breeding programs for low-input systems are likely to accelerate progress by focusing on grass–legume bicultures.

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1. Introduction

In the Cedar Creek biodiversity experiment in Minnesota, USA, high-diversity plots consisting of 16 plant species have had, on average, biomass yields exceeding those of less diverse plots composed of randomly selected grassland species (Tilman et al., 2001, 2006a). Because the highest diversity plots were most productive and required few inputs, the authors concluded that high plant diversity systems are worthy of expanded research as a means to achieving carbon-negative biofuels. Fertilized monocultures of *Panicum virgatum* (one of the species in the Tilman et al., 2006a study) also have the potential to produce high biomass (Schmer et al., 2008), but any meaningful comparisons of yields from low-input high-diversity systems versus fertilized monocul-

ture systems awaits side-by-side studies that control for soil, climate, and other site-specific effects, especially since the study of Tilman et al. (2006a) was performed on an extremely nutrient-depleted sandy soil.

Breeding of high-yielding varieties of *P. virgatum* for use in fertilized monocultures is under way, which is expected to raise the yield of the high-input system beyond its current level (Schmer et al., 2008). An important question to now consider is whether biofuel crop breeding programs should also be initiated for low-input systems. The feasibility of breeding for a low-input system is dependent on the level of plant species diversity required in the system. Strategies for selection in intercrop systems have been devised, but they are generally complex (Wright, 1985; Griffing, 1989). Not only do species and genotypes interact, but planting densities, harvest dates, locations, and years will produce further interactions. The result is that plant breeders have rarely attempted to breed for mixtures of two species, and breeding programs explicitly for high-diversity species mixes do not exist to

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our knowledge. It seems unlikely that plant breeders will undertake breeding programs for low-input systems if high species diversity (for instance, randomly arranged 16-species mixtures) is essential to the success of those systems.

Agricultural scientists, particularly forage agronomists, have investigated the potential for biomass yield increase through increasing plant species numbers. In their review of plant species diversity effects in forage and grazing lands, Sanderson et al. (2004) describe the evolution of thought regarding plant species diversity in this field. Early research advocated complex species mixtures. Emphasis shifted during the 1950s to a maximum of one grass and one legume species, combined with management to sustain the legume. Ecological studies of grassland species diversity since that time have resulted in renewed interest in forage plant species diversity. In a striking example, the results of the BIODEPTH project (a cross-European study of the effect of biodiversity on the functioning of grassland ecosystems) showed a log-linear reduction in yield due to species loss and were extrapolated to predict 100 million Swiss francs lost for every halving of species diversity in the grasslands of Switzerland (Minns et al., 2001). Although the impact of random species loss is an interesting theoretical consideration, it does not provide the basis of a sound prescriptive approach to agriculture.

Knowledge of the particular species and functional groups present in each plot can also be used to make inferences about the relative importance of species or functional group identity on ecosystem properties. In the case of the BIODEPTH experiment, for instance, each halving of the number of plant species reduced productivity by approximately $80\ g\ m^{-2}$, whereas omitting the single nitrogen fixing species *Trifolium pratense* on average reduced productivity by $360\ g\ m^{-2}$ (Hector et al., 1999). Stated in agricultural terms, adding *Trifolium pratense* to a grass monoculture would on average increase the yield similarly to adding up to 11 species selected at random.

One strength of species diversity studies such as the Cedar Creek and BIODEPTH projects is the mostly random manner in which species or functional groups are selected for each species diversity treatment. This design allows inference about the effect of species or functional group presence *per se*. By comparison, agronomic experiments typically only consider the addition of species to a consistently high-yielding low-diversity mix or monoculture. This common agronomic approach answers the question of whether farmers are justified in purchasing a diverse mix of seeds versus a less expensive low-diversity mix, but provides little information about the basic biology of the species or system in question.

A recent round of experiments has been conducted by forage agronomists to determine the minimum number of species required in a plot to maximize yield. In a comparison of mixtures of three, five, and eight forage species sown in 3–6-ha pastures, forage yield and cow–calf performance were unaffected by species number (Tracy and Faulkner, 2006). An experiment with pasture species diversity ranging from 1 to 15 species indicated that maximum forage yield and stability will likely be achieved with two to three species that are well matched to the environment rather than by planting a random assemblage of many species (Tracy and Sanderson, 2004). In contrast, a comparison of plots with seven grasses versus plots with 11 grasses and 28 forbs has shown consistently higher yields in the diverse plots, with a 43% higher hay yield in the eighth year (Bullock et al., 2007). Similarly, an 11-species forage mix outyielded a two-species mix by 43% over 4 years (Skinner et al., 2006). However, when the higher diversity treatments have greater yield, experimental designs that compare only fixed sets of species provide no information about the minimum number of species required for high yield. Possibly, a small number of species

from the high-diversity mix might have given yields similar to that of the high-diversity mix.

Cardinale et al. (2007) performed a meta-analysis of 44 different experiments that studied the effect of plant species richness on biomass production for various lengths of time. They found that the likelihood of transgressive overyielding increased through time. The results indicated that it takes about 5 years, on average, for the most diverse polyculture to have unambiguous evidence of overyielding, and only Tilman's (2001) experiment had more than 5 years of data. An analysis of this same experiment which included data from 10 years showed that complementarity effects were significant and growing in the third through tenth years of the experiment (Fargione et al., 2007).

Given that complementarity effects are strong in the later years of Tilman's (2001) experiment (Fargione et al., 2007), monocultures in this low-input system are certain to have yields substantially lower than the most diverse polycultures. Now we ask the question critical to plant breeders and agronomists: if monocultures are inadequate to achieve maximum sustained yield in low-input systems, what is the minimum species number required to achieve aboveground biomass production and yield stability that are indistinguishable from that of the highest diversity treatments? Secondarily, are particular species or functional groups necessary to achieve maximum biomass yield with low input? With preliminary answers to these questions, plant breeders and agronomists will know where to begin in the further development of low-input production systems.

2. Materials and methods

We controlled plant species number in 168 plots sized 9 m by 9 m at the Cedar Creek Natural History Area, MN, USA. Plots were randomly seeded with 1, 2, 4, 8, or 16 perennial grassland species, the composition of each plot being chosen at random from a set of 18 species. All plots were seeded at a rate of $10\ g\ m^{-2}$ in May 1994 and again treated with $5\ g$ seed m^{-2} in May 1995. Seed mass was divided equally among species in each plot. Plant species number treatments were maintained by weeding two to four times per year. Weeds were removed while they were small, taking care to minimize disturbance. Plots were burned annually each spring to remove aboveground biomass. In plots containing trees among many other species, annual burning typically removed most woody biomass, and annual aboveground biomass production of the woody species was estimated. Plots dominated by woody species that did not burn annually were excluded from the analysis, leaving 152 plots with data from 1996 to 2006. Plots were sampled annually in mid-August by clipping, drying, and weighing four parallel and evenly spaced 0.1 m by 3.0 m strips per plot from 1996 to 1999, and four 0.1 m by 6.0 m strips per plot from 2000 to 2006. The position harvested in each plot was different each year. Prior to planting the experiment, soil was sampled to 20 cm depth and percent carbon content was determined.

Statistical analyses were conducted using the R statistical system, available from www.r-project.org, and widely documented (for example, Venables and Ripley (2002)), both for graphics and for model fitting. We generally fit linear mixed models, using the R package's nlme (Pinheiro and Bates, 2000) and lme4 (www.cran.r-project.org/web/packages/lme4/). To assess the significance of the species number (slope) coefficient, we used the Markov Chain Monte Carlo scheme in R's lme4 package.

3. Results

Eleven-year average biomass yield increased with the logarithm of the number of species planted (Fig. 1A). Plotting 95% marginal prediction intervals reveals the high variation among
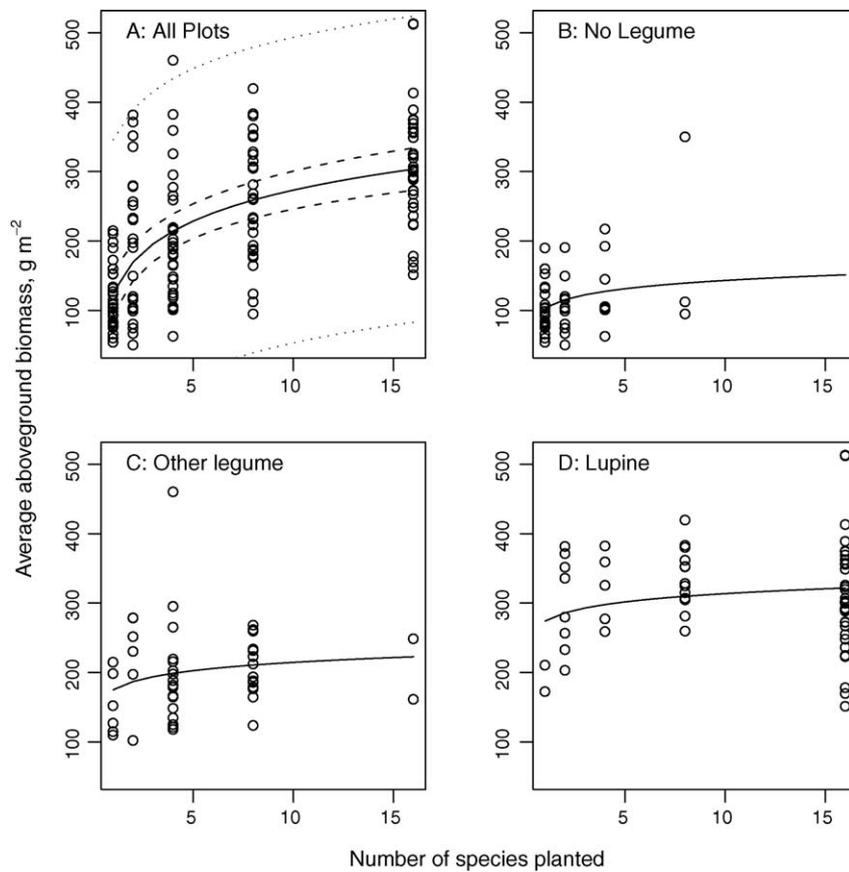
Fig. 1. (A) Log2-least-squares regression of 11-year average aboveground biomass on planted species number, with the 95% confidence interval (dashed lines) for the estimated mean and the 95% marginal prediction interval (dotted lines) for an individual plot in a given year. Log2-regressions with separate intercepts for plots with: (B) no legumes, (C) any legume besides *Lupinus perennis*, or (D) *L. perennis*.

plots planted with a given number of species. The variation may in part be due to the varied species compositions of plots with the same species number. Since making recommendations to farmers requires a narrow prediction window, the analysis that followed was motivated by the desire to use species identity information to determine the minimum species number necessary for the system to have predictably high yield.

Because legume presence and identity have had large effects on yield in diversity experiments (Hector et al., 1999; Tilman et al., 2001), we began the analysis by plotting the data with different markings for legume presence and identity. Three obvious categories emerged: plots with no legumes, plots with the legume species *L. perennis*, and plots with any legume other than *L. perennis* (Fig. 1B). Because of the overwhelming difference in these three categories of plots, we developed mixed linear models with their addition.

Model 1 (Table 1) is similar to the model of biomass energy from harvests in years 2003–2005 (Tilman et al., 2006a). Model 2 allows for three different intercepts, depending on legume/*L. perennis* presence, but only allows for a single slope based on the base-two logarithm of species number. Using the estimated coefficients of Model 2, the estimated increase in yield for adding *L. perennis* to a plot with no legume present is the difference in intercept between plots with no legume and plots with *L. perennis*, 170.1 g m^{-2} biomass, whereas each doubling of species number through random species addition (excluding the effect of *L. perennis*) would result in an average increase of 11.9 g m^{-2} biomass. Model 3 allows for separate slopes and intercepts based on legume/*L. perennis* presence and is equally good, having a slightly lower AIC (Akaike's information criterion). The species number (slope) coefficient is marginally significant in Model 3 for plots with no legumes, and nonsignificant for plots containing any legume.

Model 4 (Table 1) was the outcome of a model selection strategy wherein we considered all two-way interactions between functional groups present in the plot (C_3 grasses, C_4 grasses, forbs, and trees) and starting soil C, interacting with the three major legume groupings (none, *L. perennis*, or other legumes). This model has the lowest AIC and has 67% less unexplained plot-to-plot variance than Model 1. Model 4 indicates that doubling species number with no legume or any legume other than *L. perennis* is expected to increase biomass by about 21–31 g m^{-2}. In plots that contain *L. perennis*, the presence of any C_4 grass in these plots will on average increase biomass by about 54 g m^{-2}, but random species additions are not expected to give an added increase in biomass. Soil C had a positive interaction with C_4 grass presence in all three legume groupings.

Models 2 through 4 allow year-to-year variance to differ according to legume/*L. perennis* presence; estimated year-to-year variance components are the same for all these models because REML (restricted maximum likelihood) was used to obtain estimates. The year-to-year variance was an order of magnitude larger in plots with *L. perennis* than in plots with no legume, and more than twice that of plots with legumes other than *L. perennis*. The cause of the increased variance in plots with legumes, and particularly *L. perennis*, was the relatively large increase in yield of these plots during the first 6–7 years of the experiment (Fig. 2A), while yields in plots without legumes were consistently low. We also fit models allowing year variance to depend on the number of planted species, and obtained similar results because biomass in plots with higher species number increased over the first half of the period (Fig. 2B). Models with year variance depending on legume status had slightly lower AIC values, but the random assignment of species to plots confounds legume status and species number, so differential effects of these two factors cannot be determined.

Table 1
REML estimates from four mixed linear models of aboveground biomass over 11 years: Model 1 assumes constant variance with a single slope and intercept, but with random plot and year effects, Model 2 includes a single slope and three intercepts, Model 3 allows for three intercepts and three slopes, and Model 4 accounts for complex interactions between *Lupinus perennis*/other legume presence, C_3 grass (C_3) and C_4 grass (C_4) functional group presence, species number, and starting soil carbon.

	Model 1	Model 2	Model 3	Model 4
AIC[a]	19240	18968	18958	18843
Random effect variance[b]				
Plot	6632	3775	3809	2181
Year	1254			
No legume		264	264	264
Other legume		1212	1212	1212
L. perennis		3511	3511	3511
Residual	4530	3931	3931	3931
Coefficient estimates				
Intercept	**125.3 ± 16.0[c]**			
Log2 (species number)	**44.5 ± 4.7**	**11.9 ± 4.8**		
No legume		**103.9 ± 11.4**	**97.5 ± 13.5**	**113.1 ± 15.1**
No legume × Log2 (species number)			19.9 ± 10.3	**25.9 ± 11.3**
No legume × C_3				−28.9 ± 20.0
No legume × C_4				−11.2 ± 17.2
No legume × carbon				0.2 ± 0.6
No Legume × C_4 × carbon				**2.2 ± 0.9**
Other legume		**175.0 ± 17.2**	**177.7 ± 22.9**	**180.5 ± 19.3**
Other legume × Log2 (species number)			10.6 ± 9.0	**31.3 ± 10.7**
Other legume × C_3				**−70.8 ± 21.8**
Other legume × C_4				16.7 ± 17.6
Other legume × carbon				−0.3 ± 0.7
Other legume × C_4 × carbon				**4.8 ± 1.2**
L. perennis		**274.0 ± 24.7**	**282.7 ± 29.1**	**269.1 ± 25.9**
L. perennis × Log2 (species number)			9.1 ± 6.9	3.1 ± 10.6
L. perennis × C_3				−19.9 ± 25.2
L. perennis × C_4				**53.8 ± 23.9**
L. perennis × carbon				−1.2 ± 1.9
L. perennis × C_4 × carbon				**4.2 ± 1.9**

[a] AIC, Akaike's information criterion, a model selection statistic that includes a penalty for the number of parameters in the mean function to avoid over-fitting. Lower values indicate a better model.

[b] Models 2–4 allow separate year-to-year variances depending on presence of *L. perennis*, any other legume, or no legume in a plot.

[c] Significant ($P < 0.05$) effects are in bold, and ±values indicate standard errors.

We fit models including fixed quadratic time trends (data not shown) but prefer to consider year as a random effect because in an agricultural context yield variation *per se* matters more than variation around a curve that could be fit. In this case, the quadratic response could be due primarily to dry weather conditions in the later years of the experiment, and the trend may not continue in coming years.

To examine the effect of species number on yield stability across years, we modeled biomass yield in 16- and 2-species plots with *L. perennis* and a C_4 grass (according to Model 4, *L. perennis* and a C_4 grass are necessary for maximum biomass). Then we compared the selected 2-species plots with 16-species plots in each year. In the first 2 years, biomass was greater in 2-species plots than 16-species plots ($P < 0.05$), but biomass of the 2- and 16-species plots was similar in later years. Because the 16-species plots also had their species compositions determined randomly, it is plausible that some subset of these plots might outperform the selected 2-species plots, but any difference would be small.
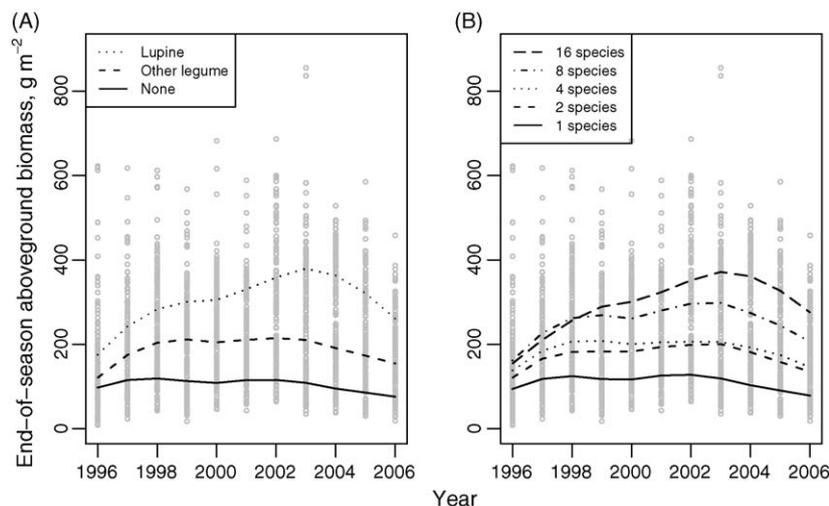

Fig. 2. (A) End-of-season aboveground biomass for each plot versus year, with separate loess smooths for the three legume groups: no legumes (none), any legume besides *Lupinus perennis* (other legume), or with *L. perennis* (lupine). (B) Aboveground biomass for each plot versus year, with separate loess smooths for each number of species planted.

4. Discussion

We have presented a range of models that vary in complexity. The simplest model offers the most robust conclusion because it is consistent with the original experimental design—aboveground biomass increases in a log-linear fashion with increasing planted species number. The more complex have the risks inherent in a post hoc analysis. However, clear patterns emerge from Models 2–4 (Table 1): some 2-species plots produce aboveground biomass yields similar to the most species-diverse plots, and legume presence and identity strongly influences aboveground biomass yield.

The most complex model, Model 4, accounted for the variability of starting soil carbon and included some key interactions. This model was clearly superior to other models, in having the lowest AIC and only one-third the unexplained plot-to-plot variance of Model 1. The increased complexity of Model 4 allowed the identification of several substantial effects. For instance, higher biomass yields were obtained in plots containing a particular species, *L. perennis*, and at least one C_4 grass. Therefore, at least two species were required for near-maximum biomass yields. In the first 2 years, 2-species plots with *L. perennis* and a C_4 grass yielded more biomass than 16-species plots, but were similar in later years to the full set of 16-species plots. Although yield was initially higher in these 2-species plots, a likely explanation is that the seeding rate of *L. perennis* in the 16-species plots was one-eighth that planted in the 2-species plots, requiring several years to build plant populations and/or soil nitrogen through biological fixation to levels similar to those in the 2-species plots. This dilution effect of important species should be considered when recommending species number increases in agricultural production fields.

In lower diversity plots that did not have *L. perennis* but that did contain a different legume, adding C_3 grass species produced a negative effect on harvested biomass (Model 4, Table 1). The negative effect of C_3 grass presence was probably due to timing of harvest. Biomass in this experiment was harvested in late summer, whereas the C_3 grasses grew most rapidly in the spring and their biomass had partially decomposed by the time harvest occurred. We suggest that harvested yield may be reduced if the maturity of some species is out of sync with harvest, another concern regarding agricultural systems composed of random plant species.

The interaction between soil C and the presence of a C_4 grass depended on legume status (Model 4, Table 1). The interaction was stronger in legume-containing plots than in plots without legumes. Available water content is an important factor that is associated with soil organic matter (Hudson, 1994), and this relationship may explain the soil C by C_4 grass interaction.

We did not consider the possibility of different models for different periods of the study, even though we know complementarity effects are increasing over time in the experiment (Fargione et al., 2007). From an agricultural perspective, the 11-year timeframe of the current study is an appropriate duration to consider as a whole. Although effects in the later years of the experiment may be most relevant to those considering natural ecosystems, farmers would be more likely to give similar relevance to all years of the experiment, with a bias toward the earlier years when a rapid return on establishment costs is critical. Therefore, the models we obtained have particular relevance for agriculture, but may be of limited use for those considering the ecological effects of species number *per se*.

4.1. Comparisons to other studies

Analyzing this planted species diversity experiment using an agricultural approach reveals some agreement between its results and studies of intercropping in agriculture. In the absence of strong facilitation (usually through nitrogen fixation), intercropping yield advantages are seldom obtained in agricultural crops (Trenbath, 1976). This generalization does not apply to intercropping plant species that are genetically limited in size or rooting volume, or in situations where nutrients, disease, or pests strongly limit yield (Loomis and Connor, 1992; Li et al., 2007). Model 4 (Table 1) shows that, for this experiment, if a plot contains *L. perennis* (presumably well-adapted to this site and capable of high N-fixation rates) and a C_4 grass, random species additions did not provide further yield benefits. By comparison, in plots with other legumes or no legumes, random species addition has a strong effect on aboveground biomass. In these cases, species number is likely to be overcoming things that could limit monoculture productivity, such as low N-fixation levels, small plant size, loss to diseases or insects, or limited rooting volume.

Had *L. perennis* been omitted from this experiment, high species diversity would have appeared essential to achieving maximum biomass. Indeed, just such a result occurred in a Dutch biodiversity experiment with herbaceous perennials in which no legumes were included in the species pool (Van Ruijven and Berendse, 2003, 2005). Conversely, if the site had much higher available soil N, grass monocultures might have achieved maximum biomass, as has been seen in both grazing and clipping studies (Lowe and Bowdler, 1981; Cuttle et al., 1998). These factors underscore the importance in agriculture of selecting well-adapted species and genotypes and planting them in multiple environments to determine the species and genotype identity of the minimal set of species required for high yield for each type of environment. When such information is lacking, long time-scales are considered, or environments are heterogeneous, high-diversity mixtures could be a reasonable approach.

The importance of a key species for obtaining maximum biomass yield is in agreement with the numerous reports (Spehn et al., 2002; Cardinale et al., 2006; Picasso et al., 2008; Frankow-Lindberg et al., 2009). Picasso et al. (2008) studied plots seeded with one to seven species with two different harvest regimes. They found that plots containing a particular species generally achieved maximum biomass, regardless of diversity. However, the identity of the species required for maximum biomass differed depending on harvest regime. Similarly, the importance of *L. perennis* in particular is unique to the environment and techniques used in this experiment. Future analyses of diversity experiments should include a search for species that are key to maximum biomass yield.

4.2. Broader implications

Tilman et al. (2006a) proposed producing biomass for energy by growing diverse herbaceous fields with minimal inputs. Schmer et al. (2008) suggested that using N-fertilized perennial monocultures could be a superior approach, in part due to the plant breeding and management gains that can be made with a simplified system. For instance, yield gains per year from selection in *P. virgatum* have been between 1 and 7%, despite the limited resources allocated to *P. virgatum* research thus far (McLaughlin and Kszos, 2005). Although this rate of advance through breeding would be unrealistic to expect with high-diversity mixtures, it may be possible with bicultures. Bicultures could be arranged so that the legume species is evenly distributed within a field, or the legumes could be grown in patches, fed to livestock, and the manure used to fertilize the grasses. With a sustained effort to breed and develop legume/C_4 grass bicultures, plant breeders and agronomists may develop systems that are increasingly productive and mostly free from dependence on nitrogen fertilizers. By contrast, high-diversity mixtures may not be amenable to improvement through breeding, and *P. virgatum* monocultures

will remain dependent on energetically expensive nitrogen fertilizer.

If low-input highly productive perennial systems are possible with relatively low plant species diversity, plant breeders may be encouraged to develop perennial crops that will meet not only our energy needs, but our more basic needs for food. Plant breeders may be able to develop low-input perennial cropping systems that sustainably yield harvestable, storable, transportable grains containing the protein, oil, and carbohydrate on which humanity depends (Cox et al., 2006).

Although high levels of plant species diversity were not required to attain near-maximum biomass yields in this experiment, biodiversity is nonetheless valuable for numerous reasons, including year-to-year stability of production, decreased disease incidence, and increased diversity of the arthropod community (Tilman, 2000). If our findings are broadly applicable, then based on yield alone, low- to moderate-diversity perennial systems would be an appropriate focus for crop development efforts. One strategy to increase diversity overall would be to develop numerous lower diversity systems and deploy them in a patchwork arrangement to achieve landscape-level diversity. Or, farmers could be paid to add non-economic species to their fields solely to increase biodiversity. Finally, it is a plausible hypothesis worthy of testing that the addition of other selected species to improved bicultures could potentially increase their yields and the stability of these yields (Tilman et al., 2006b).

Jackson and Piper (1989) suggested that the fields of agriculture and ecology must be integrated in order to achieve solutions to agricultural problems. We have demonstrated a step forward in such integration. By applying an agriculturally relevant statistical analysis to ecological research, we can obtain information critical to the design of sustainable agricultural systems. Continued agriculturally relevant analyses of species diversity effects are needed. Particularly, other critical factors, such as nitrogen leaching, soil carbon accumulation, invasion, pathogen load, and wildlife habitat should investigated.

5. Conclusions

In the low-input grassland system we studied, one particular legume species (which was well-adapted to the site and presumably fixed nitrogen) was critical to maximum biomass production. Beyond mixing this species with any C_4 grass species, there was no evidence that additional random species additions increased average aboveground biomass or stability of aboveground biomass production in the 11-year study. Therefore, bicultures including adapted legumes appear so far to be an adequate starting point for plant breeding programs targeting low-input perennial production systems. As the science develops, the species developed for these simplified systems might later be combined into more species-diverse systems to provide expanded ecosystem functions.

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