The overseeding of two cool-season legumes (*Hedysarum coronarium* L. and *Trifolium incarnatum* L.) on switchgrass (*Panicum virgatum* L.) mature stands increased biomass productivity

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Abstract

In the Mediterranean rainfed systems, perennial warm-season grasses are profitable crops for the production of herbage as forage or feedstock for bioenergy purposes. During summer, when the production of cool-season crops is scarce, warm-season grasses can improve the productivity and stability of forage cropping systems. In Italy, switchgrass (*Panicum virgatum* L.) can be cultivated for herbage production or as energy crop. The objective of this work was evaluating if relay intercropping with cool-season legumes could be suited to convert a mature stand of switchgrass from energy to dual, energy and forage, production, together with improving the productivity and the quality of the harvestable biomass. All these things considered, a field experiment was carried out in Central Italy, on mature stands of two switchgrass varieties, Alamo and Blackwell, overseeded with two legumes: sulla (*Hedysarum coronarium* L.) and crimson clover (*Trifolium incarnatum* L.). The intercropping system was compared with fertilized and un-fertilized pure switchgrass stands. After two years of study, data showed that the intercropping increased the total above ground biomass (AGB) productivity. In the second year, the increase in total AGB production for switchgrass mixtures compared with the pure stands was greater for sulla, a biennial legume, than crimson clover.

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Key words: Sustainable intensification; Mediterranean environment; Blackwell; Alamo; relay intercropping.

Acknowledgements: the authors wish to thank Fabio Taccini and the Center of Agro-environmental Research (CIRAA) of the University of Pisa (Italy) for their valuable support in the field trial management.

Received for publication: 5 July 2019.
Revision received: 30 September 2019.
Accepted for publication: 3 December 2019.

Italian Journal of Agronomy 2020; 15:1510
doi:10.4081/ija.2020.1510

Introduction

Future cropping systems have to meet several goals simultaneously: i) improving productivity of croplands and grasslands in order to produce food for increasing world population (Lutz *et al*., 2001); ii) reducing global environmental impact; iii) enhancing land-use efficiency; iv) mitigating climate changes through the reduction of greenhouse gases (GHG) emissions; and v) setting up adaptation strategies to reduce the vulnerability of crops by a sustainable intensification (Campbell *et al*., 2014; Godfray and Garnett, 2014; Pretty and Bharucha, 2014; Wezel *et al*., 2014). Several authors reported that the introduction of perennial crops, through the conversion from croplands to grasslands, can contribute to increase the sustainability of agricultural production (Glover *et al*., 2010, 2012). In fact, perennial crops can provide several ecosystem services such as: i) maintaining soil fertility; ii) increasing the soil carbon stock potential compared to annual crops (Glover *et al*., 2010; Monti, 2012); iii) enhancing soil protection by all-year-round vegetation cover and contrasting soil erosion (Durán Zuazo and Rodríguez Pleguezuelo, 2008; Vallebona *et al*., 2016); iv) improving biodiversity in farmland and guaranteeing higher resilience of the agro-ecosystem (Peyraud *et al*., 2014).

In the Mediterranean, perennial warm-season grasses are profitable crops for the production of herbage as forage or feedstock for bioenergy utilization (Monti *et al*., 2012). In addition, in summer dry period when the conventional production, as pasture or fodder, is poor, warm-season grasses can improve the productivity and the stability of Mediterranean forage cropping systems (Gherbin *et al*., 2007). Among perennial warm-season grasses, switchgrass (*Panicum virgatum* L.), a prairie species, native of North America and originally domesticated for pasture production, was recognized for its ability of accumulating large amount of biomass even under drought conditions (Monti *et al*., 2012). Starting from the 90s, owing its high yield potential, switchgrass has been introduced in Europe as promising crop for bioenergy sector (Parrish *et al*., 2005). In the last decade, several studies highlighted the suitability of both lowland and upland ecotypes to Mediterranean environments (Monti *et al*., 2008, 2012; Alexopoulou *et al*., 2015; Nassi o Di Nasso *et al*., 2015). Furthermore, the literature evidenced that: i) switchgrass productivity decreases after the third year of cultivation (Alexopoulou *et al*., 2015); ii) multi-harvest systems (more than one cut per year) negatively affect the plantation life span (Monti *et al*., 2008); iii) switchgrass yields are affected by nitrogen (N) fertilization (Nassi o Di Nasso *et al*., 2015). Concerning the response of this crop to N fertilization, Ashworth *et al*. (2015b) indicated that sustainable production levels (evaluated using an life cycle assessment approach) could be achieved with low N fertilization rate (67 kg ha^{-1}), since higher amount of N decreases the efficiency of the cropping system. Moreover, the same authors proposed the intercropping with legumes to meet crop's nitrogen requirements by N fixation. Intercropping has been defined as the coexistence of two or more crops in the same field at the same time, while in relay intercropping, crops are grown together only for part of their life cycles (Vandermeer, 1989). Indeed, intercropping is an agro-ecological practice aimed to improve the sustainability of production through the establishment of facilitation processes between crops (Wezel *et al*., 2014). Wang *et al*. (2010) highlighted the possibility of the intercropping with legumes as an environmentally friendly N source for switchgrass cultivation as biofuel energy, indicating the harvest time and the management as key factors for switchgrass growing and legumes persistence. At present, only few recent researches mainly conducted in the USA have investigated the intercropping of switchgrass with cool-season legumes (Bow *et al*., 2008; Butler *et al*., 2013; Ashworth *et al*., 2015a). Recently in the Mediterranean, preliminary data of alfalfa (*Medicago sativa* L.) and switchgrass mixture showed an increase of biomass production in mixture than in pure grass stand (Mantino *et al*., 2016). Overall, the intercropping with legumes can decrease the use of N fertilizer, thus limiting the environmental risks related with nitrogen leaching and NO_x soil emissions (Anglade *et al*., 2015).

The main objective of this field study was evaluating whether the relay intercropping of cool-season legumes can be proposed as an agroecological intensification practice, for converting switchgrass mature stands from fuel to feed and fuel production in Mediterranean environment.

Thus, the present study had the following specific aims: i) investigating the suitability of the relay intercropping of two cool-season legumes, sulla (*Hedysarum coronarium* L., local ecotype) and crimson clover (*Trifolium incarnatum* L., var. Pier), with two switchgrass mature stands (Alamo and Blackwell) under Mediterranean-climate conditions; ii) assessing the response of the two switchgrass varieties to optimized harvest timing and frequency for the production of forage. To achieve these goals, a two-year field trial was carried out by overseeding the two legumes in two four-year-old pure stands of switchgrass cultivated in Central Italy.

Materials and methods

Study site description

A field trial was conducted under rainfed condition, at the Agro-Environmental Research Center "Enrico Avanzi" of the University of Pisa, located in San Piero a Grado (Pisa) (43.667205 N, 10.313160 E) for two consecutive years (2014-2015). Soil texture was clay-loam, the organic matter content was 18 g kg^{-1}, pH was 8.1, total nitrogen content was 1.3 g kg^{-1}, available phosphorus content was 35 mg kg^{-1} and exchangeable potassium was 200 mg kg^{-1}. In May 2009, two switchgrass cultivars, an up-land ecotype and a low-land ecotype, namely Alamo and Blackwell, were sown (20 kg ha^{-1} seed). The previous crop was durum wheat. In the years before the trial establishment (2010-2012), the two stands had been harvested annually during winter season with the exception of 2009, when the crop was not harvested. The average aboveground biomass yield was 20 Mg ha^{-1} dry matter (DM) for Alamo and 15 Mg ha^{-1} DM for Blackwell. Starting from 2013, a part of the two switchgrass stands had been harvested in October, in order to perform the overseeding with the two cool-season legumes. On 15th October 2013, sulla (*Hedysarum coronarium* L.), a biennial cool-season legume, and crimson clover (*Trifolium incarnatum* L.), an annual legume species, were overseeded by hand-broadcasting in the early harvested switchgrass stands, at 25 and 30 kg ha^{-1} seed, respectively. The following year, on 12th October 2014 the crimson clover was overseeded again in the same plots with the same seed dose.

Experimental design

The experimental design was a split plot with three replicates (20 m^2 each), where the two switchgrass varieties, Alamo and Blackwell, represented the two levels of the main factor (V, variety), while the sub-factor (S, system) included six different treatment levels. There were:

- 2 intercropped systems: i) *Ssu* - switchgrass intercropped with sulla; and ii) *Sc* - switchgrass intercropped with crimson clover.
- 2 control systems, in which the pure switchgrass stands were cut a first time, in early season, in correspondence of the harvesting of the legume crop of the reference intercropped system: iii) *S0-su* - unfertilized pure switchgrass harvested in same dates of *Ssu*; and iv) *S0-c* - unfertilized pure switchgrass harvested in same dates of Sc.
- 2 pure stand switchgrass systems: v) *S50* - pure switchgrass fertilized with 50 kg ha^{-1} N; and vi) *S0* - unfertilized pure switchgrass harvested in same dates of S50 (Table 1).

In Ssu, Sc, S50 and S0, harvest timing and frequency were managed in order to optimize the forage yield and quality. Indeed, the intercropped systems (Ssu and Sc) were harvested three times per year: i) in spring, at legume early flowering stage; ii) in summer, at switchgrass flowering stage; iii) in autumn, at the end of growing season (in the case that a regrowth was observed). S0 and S50 were harvested two times: in summer, at flowering stage and in autumn (in the case that a regrowth was observed). The control systems, S0-su and S0-c, were harvested according to Ssu and Sc, in order to evaluate the effect of an early cutting on the pure switchgrass stands. In S50, N fertilization was performed two weeks after switchgrass re-sprouting (10th and 15th April in 2014 and 2015, respectively).

Data collection

In 2014 and 2015, the crops were sampled on a 1 m^2 area per each sub-plot at each harvest time, as reported in Table 1. The cutting height was set at 5 cm above the soil level. In the sub-plots, border plants were excluded from sampling to avoid field margin effect. For each sample, the total fresh weight was recorded and then the fresh biomass of switchgrass, legumes and weeds were weighted separately. Subsamples of each crop were placed in a forced-draft oven at a 60°C until constant weight for the determination of the DM content in order to determine the above-ground biomass (AGB) of switchgrass and legumes at each cut. A meteorological station, located within 1000 m distance, was used to collect daily values of air temperature and rainfall.

Statistical analysis

The annual yield of each system (total annual AGB) was computed as the sum of the AGB sampled at each harvest time. The statistical analysis was performed using the R software (R Core Team, 2018). For all annual AGB data, Bartlett's test was used to test the homogeneity of variance and Shapiro-Wilk test to confirm normality of residuals. Data transformation was not necessary. The effect of Year (Y), Variety (V) and System (S) on total, switchgrass and legume annual AGB was determined using the lmer() function for linear mixed-effects models, in the "lme4" package (Bates *et al*., 2014), with the factors Y, V and S used as fixed effects and Y as a random effect. Tukey's HSD post-hoc test was carried out by pairwise multiple comparisons using the "emmeans" package (Lenth 2019) with the emmeans() function (α=0.05).

Results

Meteorological data

Meteorological data, about the reference period (October 2013 - September 2015), are reported in Figure 1. In the first year (October 2013 - September 2014) total rainfall was about 1200 mm, 36% higher than the long-term average (from 1971 to 2000). A similar value (1100 mm) was recorded in the following year (October 2014 - September 2015) with an increase of precipitation of about the 23% respect to the long-term average. In particular, during autumn 2013 (October-December) rainfall was lower than the long-term average (194 *vs* 338 mm), conversely in the same period of 2015, the rainfall was about 58% higher. During winter (January-March) rainfall increased of about 300% and 150%, in 2014 and 2015 respectively, compared to the long-term average (181 mm). In both years, spring (April-June) precipitations were lower than the long-term average, with a reduction of 65% and 35% in 2014 and 2015, respectively.

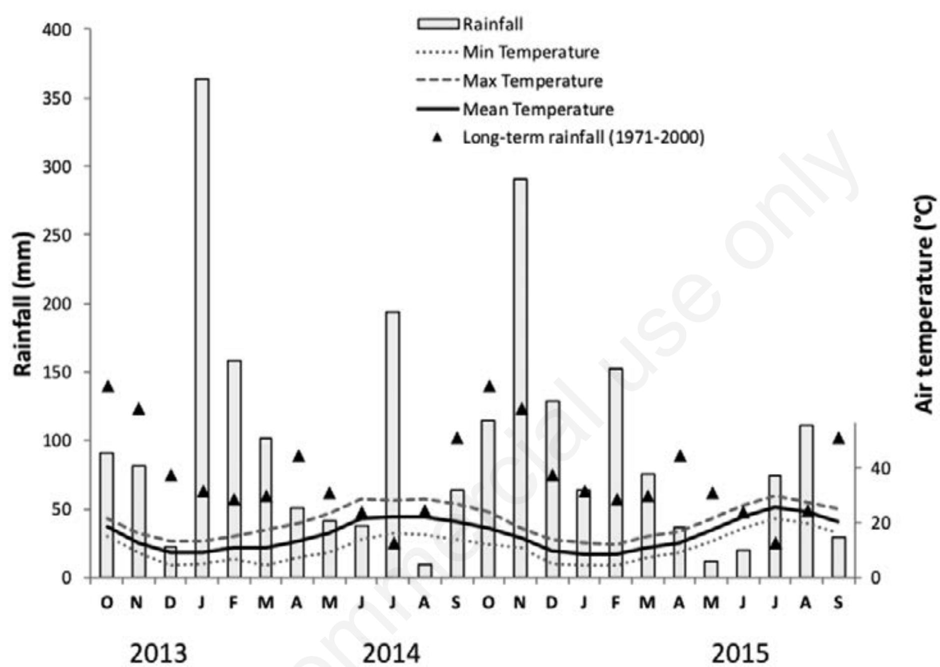

Figure 1. October 2013 - September 2015 and long-term 1971-2000 monthly rainfall, minimum, average and maximum monthly air temperature in San Piero a Grado (Pisa, Italy). The graph is presented as a Bagnouls and Gaussen (1957) diagram, in order to identify dry months, *i.e.* when rainfall (R) is equal to or lower than twice the monthly mean air temperature value (T) (R≤2T).

Table 1. Harvest dates of the six analysed cropping systems: switchgrass intercropped with sulla (Ssu), unfertilized pure switchgrass harvested in same dates of Ssu (S0-su), switchgrass intercropped with crimson clover (Sc), unfertilized pure switchgrass harvested in same dates of SC (S0-c), pure switchgrass fertilized with 50 Kg N ha^{-1} (S50) and unfertilized pure switchgrass harvested in same dates of S50 (S0).

			2014	
Variety	System	Spring harvest	Summer harvest	Autumn harvest
Alamo	Ssu / S0-su	Apr-29	September-8	-
	Sc / S0-c	Apr-15	September-8	-
	S50 / S0	-	September-8	-
Blackwell	Ssu / S0-su	Apr-29	July-31	October-9
	Sc / S0-c	Apr-15	July-31	October-9
	S50 / S0	-	July-31	October-9
			2015	
Variety	**System**	**Spring harvest**	**Summer harvest**	**Autumn harvest**
Alamo	Ssu / S0-su	Apr-24	September-1	October-5
	Sc / S0-c	May-5	September-1	October-5
	S50 / S0	-	September-1	October-5
Blackwell	Ssu / S0-su	Apr-24	August-3	October-5
	Sc / S0-c	May-5	August-3	October-5
	S50 / S0	-	August-3	October-5

Conversely, much higher rainfall levels were observed during summer (July-September) compared with the long-term average. Indeed, in 2014, summer rainfall amounted at 267 mm (+51%), while in 2015 it was 214 mm (+21%). The driest months were August (9 mm rain) and May (12 mm) in 2014 and 2015, respectively. Concerning air temperature, the average temperature (October 2013-October 2015) was 16°C, with maximum and minimum average values of about 21°C and 11°C, respectively. The coldest months were March (4.6°C average Tmin) and January (4.6°C), in 2014 and 2015. The highest temperatures were reached in June 2014 (28.7°C average Tmax) and July 2015 (29.6°C).

Annual aboveground biomass production

Results of statistical analyses of total, switchgrass and legumes annual AGB are showed in Table 2.

In 2015, the average total AGB of the tested systems was significantly lower than 2014, while there were not significant differences on total, switchgrass and legumes AGB between the two switchgrass varieties. Differently, the total annual AGB significantly varied (P<0.001) among the six systems (S) as well as the switchgrass AGB (P=0.005). The two-year-average of total Ssu AGB was the highest (13.5 Mg ha^{-1} yr^{-1}), mainly due to the legume AGB (Figure 2). Total AGB of Sc was 30% less than Ssu, and it was similar to that of the two pure systems, S0 and S50. Averaging among the six systems, switchgrass AGB was about 8.4 Mg ha^{-1} yr^{-1}, and it did not show significant differences between intercropped systems (Ssu and Sc) and the control switchgrass systems (S0-su and S0-c). Moreover, we did not observe a significant response of switchgrass to N application, being S50 comparable with S0 (Figure 2). Productivity of unfertilized switchgrass was lower than S50 when managed with multiple harvests (S0-su and S0-c). Similarly, annual AGB in legumes varied significantly (P<0.01) between the two intercropped systems (Table 2). The different productivity of the two intercropped systems was mainly due to sulla and crimson clover AGB which was in average 5.1 and 2.4 Mg ha^{-1} yr^{-1}, respectively. The first order interaction Y × V affected both the total and the switchgrass AGB (P<0.001), but not the legumes one.

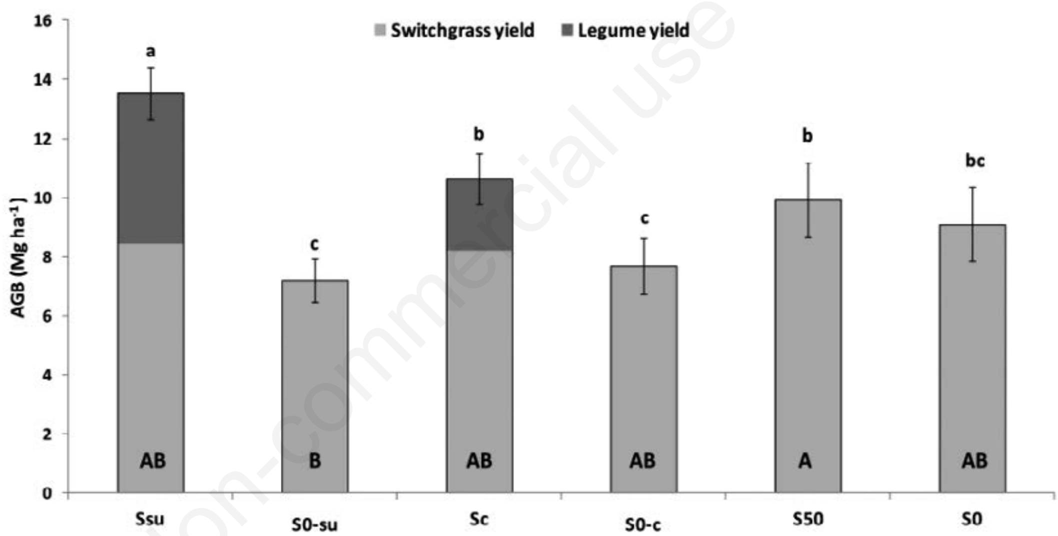

Figure 2. Two-year average above-ground biomass (AGB) yield of the six analysed cropping systems (sulla [Ssu], crimson clover [Sc], fertilized [S50] and the relative unfertilized systems S0-su, S0-c and S0). Different letters indicate significant difference, P<0.05, for Tukey's honestly significant difference test. Different uppercase letters indicate differences in the total annual switchgrass yield among the cropping systems, while different lowercase letters indicate differences in the total annual yield among the cropping systems. Vertical bars represent standard errors.

Table 2. P values of the main effects and interactions of total annual, switchgrass annual and legume annual above-ground biomass yield.

Source of variation	df	Total annual AGB	Switchgrass annual AGB	df	Legume annual AGB
Year (Y)	1	*0.0170*	0.2243	1	0.7709
Variety (V)	1	0.9834	0.5766	1	0.1301
System (S)	5	*<0.0001*	*0.0046*	1	*<0.0001*
Y × V	1	*<0.0001*	*<0.0001*	1	0.3764
Y × S	5	*<0.0001*	*<0.0001*	1	*0.0020*
V × S	5	0.5455	0.3661	1	0.9552
V × S × Y	5	0.2224	0.2647	1	0.2396

AGB, above-ground biomass; df, degree of freedom. Values in italic are for P<0.05, indicating significant differences among levels.

In the Blackwell-based systems, the decrease of total AGB from 13 to 6.3 Mg ha^{-1} yr^{-1} was observed in 2014 and 2015, respectively (Figure 3A), due to a significant reduction (about 59%) of switchgrass AGB. Differently, in the Alamo-based systems, the total AGB was similar in the two years with an average production of 9.7 Mg ha^{-1} yr^{-1}. The first order interaction Y × S affected total, switchgrass and legumes annual AGB. The total AGB of all systems, with the exception of Ssu, decreased from 2014 to 2015 (Figure 3B). Indeed, from 2014 to 2015, total AGB of Ssu increased of about 22%, while in the other systems total AGB decreased in average of about 47%. In 2014, Ssu showed a total AGB that was 2.9 Mg ha^{-1} higher than the control S0-su, while, in 2015, the difference between the two systems was larger, with Ssu producing 9.8 Mg ha^{-1} more than its control. In both years, the total AGB was higher in Sc than in S0-c, and the reduction observed from 2014 to 2015 was more evident in the intercropped system than in the pure switchgrass control (–36% for Sc and –46% for S0-c). The largest reductions from 2014 to 2015 were observed in both fertilized and unfertilized pure stands of switchgrass (–53% and –51% for S50 and S0, respectively). The positive effect of intercropping with sulla was observed, in particular, in Alamo-based systems, where total AGB of Ssu increased from 6.8 to 11.2 Mg ha^{-1}, while Blackwell-based systems, total AGB of Ssu decreased from 9.2 Mg ha^{-1} to 6.6 Mg ha^{-1}, from 2014 to 2015. In 2014, switchgrass AGB in Alamo-based systems (Table 3) aver-

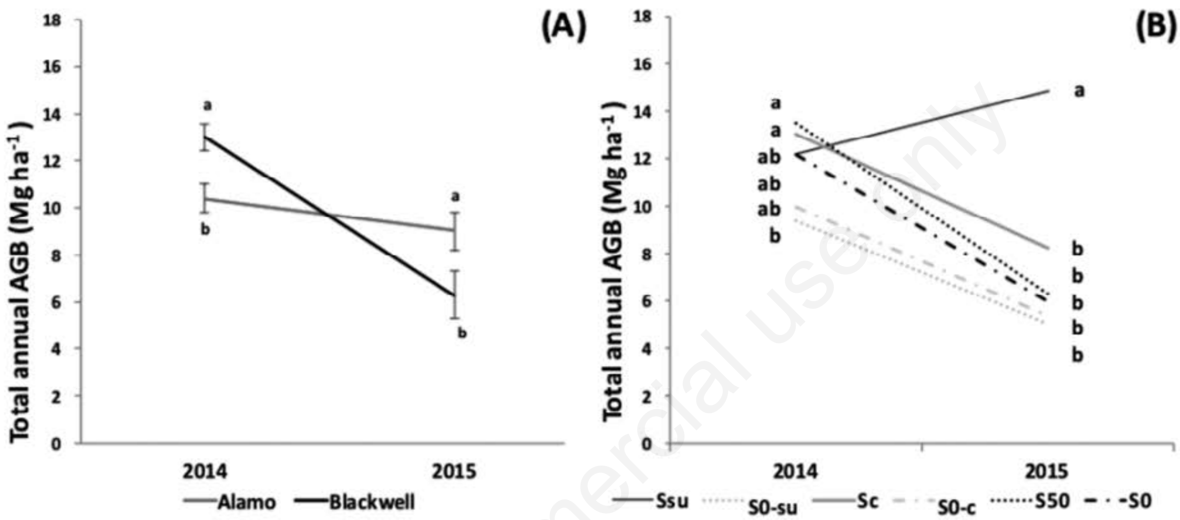

Figure 3. A) Total annual above-ground biomass (AGB) yields between varieties in 2014 and 2015. Different letters indicate significant differences for the V × Y interaction, P<0.05, for Tukey's honestly significant difference (HSD) test. Vertical bars represent standard errors. B) Total annual AGB among the six cropping systems: switchgrass intercropped with sulla (Ssu), pure unfertilized switchgrass harvested in same dates of SSu (S0-su), switchgrass intercropped with crimson clover (Sc), pure unfertilized switchgrass harvested in same dates of SC (S0-c), pure switchgrass fertilized with 50 Kg N ha^{-1} (S50) and pure unfertilized switchgrass harvested in same dates of S50 (S0) in 2014 and 2015. Different letters indicate significant differences for the S × Y interaction, P<0.05, for Tukey's HSD test.

Table 3. Switchgrass, legume and total annual above-ground biomass yields (Mg ha^{-1}) of the six cropping systems: switchgrass intercropped with sulla (Ssu), pure unfertilized switchgrass harvested in same dates of Ssu (S0-su), switchgrass intercropped with crimson clover (Sc), pure unfertilized switchgrass harvested in same dates of Sc (S0-c), pure switchgrass fertilized with 50 Kg N ha^{-1} (S50) and pure unfertilized switchgrass harvested in same dates of S50 (S0). Reported data refer to the two varieties (Alamo and Blackwell) in the 2014 and 2015 growing season.

		2014			2015		
Variety	System	Switchgrass AGB	Legume AGB	Total AGB	Switchgrass AGB	Legume AGB	Total AGB
Alamo	Ssu	6.78^{b}	3.76^{a}	10.54^{a}	11.21^{a}	5.66^{a}	16.87^{a}
	S0-su	9.88^{ab}	-	9.88^{a}	6.18^{b}	-	6.18^{b}
	Sc	8.81^{ab}	3.31^{a}	12.11^{a}	8.00^{ab}	0.87^{b}	8.87^{b}
	S0-c	7.77^{ab}	-	7.77^{a}	6.74^{ab}	-	6.74^{b}
	S50	11.85^{a}	-	11.85^{a}	7.84^{ab}	-	7.84^{b}
	S0	10.01^{ab}	-	10.01^{a}	7.26^{ab}	-	7.26^{b}
	Mean	9.18	3.50	10.36	7.87	3.27	8.96
Blackwell	Ssu	9.24^{c}	4.63^{a}	13.87^{a}	6.58^{a}	6.25^{a}	12.83^{a}
	S0-su	8.82^{c}	-	8.82^{b}	3.85^{a}	-	3.85^{b}
	Sc	10.86^{bc}	3.04^{a}	13.90^{a}	5.13^{a}	2.49^{b}	7.62^{b}
	S0-c	12.14^{abc}	-	12.14^{ab}	4.02^{a}	-	4.02^{b}
	S50	15.16^{a}	-	15.16^{a}	4.75^{a}	-	4.75^{b}
	S0	14.36^{ab}	-	14.36^{a}	4.71^{a}	-	4.71^{b}
	Mean	11.76	3.80	13.04	4.84	4.37	6.30

AGB, above-ground biomass. Lowercase letters represent significant differences among systems resulting from the post-hoc test.

aged 9.2 Mg ha^{-1}, with the highest and the lowest values observed in S50 (11.8 Mg ha^{-1}) and in SSu (6.8 Mg ha^{-1}). Alamo AGB in the two intercropped systems (Ssu and Sc) showed not significant differences, and the average legume AGB was 3.5 Mg ha^{-1}. All Alamo-based systems showed similar total AGB (10.3 Mg ha^{-1}, average value). During 2015, Alamo-based systems showed the highest switchgrass AGB in Ssu (11.2 Mg ha^{-1}) and the lowest in S0-su (6.2 Mg ha^{-1}), while the average was 7.9 Mg ha^{-1}. Sulla AGB was significantly higher than crimson clover AGB of about five times. Furthermore, significant differences among systems were observed also for total AGB. Indeed, Ssu production was the highest (16.9 Mg ha^{-1}) while the other systems performed similarly (7.4 Mg ha^{-1}, average value).

In 2014, in Blackwell-based systems, switchgrass AGB averaged 11.8 Mg ha^{-1}, with the lowest observed in Ssu and S0-su (9.2 and 8.8 Mg ha^{-1}) and the highest in S50 (15.2 Mg ha^{-1}). Conversely, in the 2015, switchgrass AGB was not affected by cropping system (4.8 Mg ha^{-1}). Legumes AGB showed the same behaviour previously described for Alamo-based intercropped systems. Indeed, in 2014, sulla and crimson clover AGB averaged 3.8 Mg ha^{-1}, while in 2015 sulla AGB was significantly higher than crimson clover (6.2 *vs* 2.5 Mg ha^{-1}). In Blackwell-based systems, a positive effect of relay intercropping with sulla was observed in terms of total annual AGB. Indeed, in 2014, Ssu performed better than its control system (S0-su) (13.9 *vs* 8.8 Mg ha^{-1}). In 2015, total AGB of Ssu was significantly higher than all the other systems, 12.8 *vs* 5.0 Mg ha^{-1} as the mean of all the other systems.

Description of seasonality of the biomass production according to harvest management

In this section, total, switchgrass and legumes AGB have been described according to the harvest time and frequency of the tested systems. In both years, at spring harvest, total AGB of the intercropped systems, Ssu and Sc, was higher than the controls (S0-su and S0-c) (Figure 4). We observed a larger contribution of the two legumes regardless the species, with the exception of Blackwell-based Ssu in 2014, where switchgrass AGB was slightly higher than sulla (3.3 *vs* 3.1 Mg ha^{-1}). In 2015, at spring harvest, both switchgrass varieties were not re-sprouted yet. Switchgrass AGB accumulation occurred mainly during summer and some differences between the varieties were observed. Indeed, Alamo AGB was 8.6 Mg ha^{-1} and 6.7 Mg ha^{-1} in 2014 and 2015, respectively, Blackwell AGB was 9.1 Mg ha^{-1} and 3.2 Mg ha^{-1}.

In 2014, after summer harvest, switchgrass regrowth was observed only in Blackwell, whose AGB at autumn harvest was 1.7 Mg ha^{-1} (averaged value among the systems). In 2015, Blackwell showed the same behaviour producing 1.4 Mg ha^{-1} (averaged value among the systems). In contrast, Alamo AGB for the 2014 autumn harvest was almost zero; in 2015 produced 0.6 Mg ha^{-1} (Figure 4).

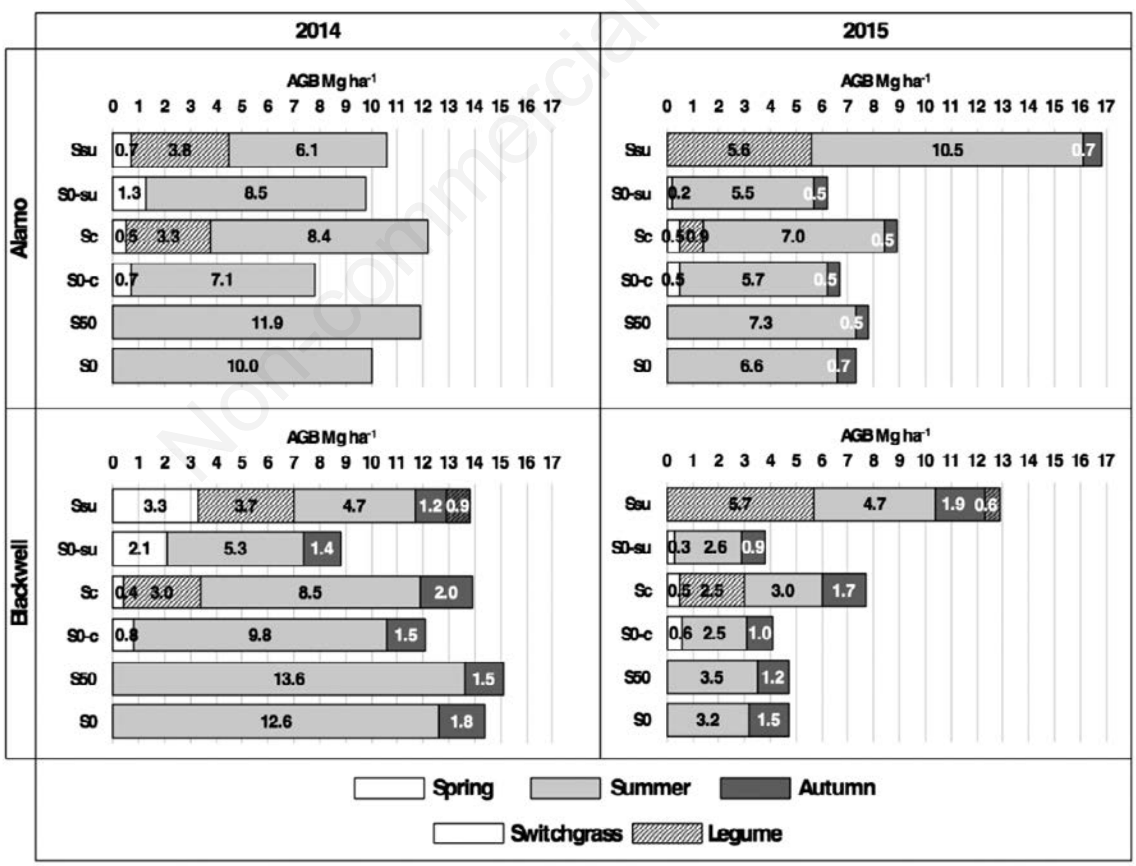

Figure 4. Above-ground biomass (AGB) yield in spring, summer and autumn harvests of switchgrass and legumes in the six cropping systems during the 2014 and 2015 growing seasons: switchgrass intercropped with sulla (Ssu), unfertilized pure switchgrass harvested in same dates of Ssu (S0-su), switchgrass intercropped with crimson clover (Sc), unfertilized pure switchgrass harvested in same dates of Sc (S0-c), pure switchgrass fertilized with 50 Kg N ha^{-1} (S50) and unfertilized pure switchgrass harvested in same dates of S50 (S0).

Discussion

During the two years of observations, meteorological conditions showed a higher variability respect to the long-term average in terms of precipitation. In both years of study, wet conditions during summer allowed the growth of switchgrass as the high rainfall during winter had a positive effect on the biomass accumulation of the two cool-season legumes.

Based on achieved results, the feasibility of switchgrass-based relay-intercropping systems needs to be evaluated considering some key topics concerning: i) the suitability of crimson clover and sulla to the pedo-climatic context; ii) the possible interactions among legumes and switchgrass; ii) the effect on switchgrass of multi-harvest, made just to achieve the best yield (in terms of quantity and quality) from the cool-season legumes.

About the feasibility of switchgrass-legume intercropping, our results agreed with those obtained in previous experiences carried out in different climate conditions of continental areas of North America (Wang *et al*., 2010). More recently, Butler *et al*. (2013) and Ashworth *et al*. (2015a) achieved similar conclusions: the relay intercropping allows to increase the total annual herbage production, being the cumulative cool-season species and switchgrass yields greater than the switchgrass monoculture. These latter also showed that the extent of the production increase depended mostly on the capability of the overseeded legumes to establish and accumulate biomass. Butler *et al*. (2013) considered five self-reseeding legumes and alfalfa, and they observed that the yield of each species was greater in the first season after planting than the subsequent years that when allowed to reseed or, in the case of alfalfa, regrow. They concluded that the reseeding model for cool-season annual legumes could not always work in switchgrass swards, suggesting the conversion from energy biomass to forage production, exploiting the protein production provided by the legumes rather than sole contribution in preserving soil fertility. In our experiment, sulla was more productive than crimson clover, and its average yield (5 Mg ha^{-1}yr^{-1}) was similar to that recorded on pure stand field trial carried out in the Mediterranean basin (Borreani *et al*., 2003; Sulas *et al*., 2003; Annicchiarico *et al*., 2014). We also observed that sulla yielded more in the second year of cultivation than in the first in both switchgrass varieties stands, complying with the same behaviour observed on a pure sulla stand in a plot trial located in southern Italy by Amato *et al*. (2016). Crimson clover was less productive than sulla, especially in the second year (2015), due to a suboptimal clover emergency, influenced by soil conditions at over-seeding and the high-intensity of rainfalls occurred after sowing, which might have decreased the germination rate due to waterlogging (November and December 2014). In the second year, crimson clover failure (producing less than 1 Mg^{-1} ha^{-1}) in Alamo was maybe due to the different habitus of Alamo that is more likely to have a bunch form with greater roots diameter which may disturb the legume establishment (Parrisch and Fike, 2005). Nevertheless, the yield of crimson clover in the two years was within the range observed by Butler *et al*. (2013) in switchgrass mixtures with Alamo.

These considerations, over our two-year-dataset, suggest that the over-seeding of cool-season annual legumes, as crimson clover, in clay-loam soil can be influenced by uncertainty of Mediterranean climate conditions during the emergence period. Butler *et al*. (2013) in a two-year field trial reported that self-reseeding annual legumes failed the regeneration in the second year and the overseeding of alfalfa needed be repeated to build up the plantation. Our observations and results reported by Butler *et al*. (2013) indicate that perennial cool-season legumes seem to be more suited than what could be expected from models based on the annual re-seeding.

During spring, the presence of cool-season legumes increases the competition for resources, in particular light and nutrient (Bow *et al*., 2008). In our study, the overlapping of grass and legumes occurred only for few days in early-spring when legumes were at flowering stage (harvest time) and switchgrass were just in re-sprouting stage. Then, our results highlighted a greater effect of sulla than crimson clover on the system performance in terms of productivity. It could be probably due to the capability of sulla of exploiting clay-loam soils more than crimson clover. However, Bow *et al*. (2008) reported a good response of switchgrass to the intercropping with another annual cool-season legume such as common vetch suggesting the need to identify more legume species to intercrop with switchgrass in Mediterranean area. Specifically, our biennial results showed a slightly, but not significant, higher switchgrass yield in intercropped systems respect to the pure control stands, probably ascribable to a higher availability of N in the soil. While the higher responsiveness of switchgrass to fixed N, could be explained with the better exploitability of fixed N along the growing season than under mineral N fertilization. This behaviour observed in our experiment was confirmed by several authors testing the mixture of switchgrass and cool-season legumes in other environment such as North America (Bow *et al*., 2008; Butler *et al*., 2013; Ashworth *et al*., 2015a).

Concerning the response of switchgrass to inorganic nitrogen fertilization, our results confirmed the low response of mature stands of switchgrass to low N supply. Similar trends were reported in studies carried out in North America on mature stands of switchgrass: no differences were reported by Butler *et al*., (2013) for a switchgrass trial fertilised with 56 kg ha^{-1} of inorganic N in a low-fertility site in Texas and by Ashworth *et al*. (2015a) in a field trial carried out in North America (Tennessee) for switchgrass fertilised with 67 kg ha^{-1} of inorganic N. Moreover, no difference between unfertilized and low-N-rate fertilized switchgrass was observed also using organic nitrogen supply in a 10-year-old stand of switchgrass fertilized with 30 Mg ha^{-1} of compost (N 9.0, P 5.6 and K 13.7 g kg^{-1}) (Bow *et al*., 2008). Furthermore, the interaction between system and year showed a strong AGB reduction of switchgrass in all the pure systems (–50%) during the second year. Conversely, in the intercropped systems the decline AGB was lower, in the case of intercropping with crimson clover (–35%), or even drastically improved with sulla (+20%). The presence of legumes did not affect the AGB production of switchgrass and it allowed to enhance the biomass quality, even considering the switchgrass alone (Ashworth *et al*., 2015c). Based on these results, the proposed intercropping systems are more suited for forage, or combined feed-fuel purposes, than for the unique supply of energy biomass, as already suggested by other authors (Bow *et al*., 2008; Butler *et al*., 2013; Ashworth *et al*., 2015a). Furthermore, we also observed that the intercropped systems performed better especially in the second year, allowing to achieve significantly higher herbage production than the pure switchgrass system. In particular, a greater facilitation occurred in the sulla based system (Ssu), allowing to achieve higher legume AGB than crimson clover, and, interestingly, a higher switchgrass production in the second year, compared with the other systems. The positive effect of sulla, was particularly evident in Alamo, that in the second year increased the biomass production of about 4 Mg ha^{-1} compared to the first year. As reported in literature, we could expect a higher fixed-N exploitation by switchgrass in the second year of intercropping (Ashworth *et al*., 2015c).

Concerning the effect of harvest frequency on switchgrass, several authors have already showed that cutting two or three times per year did not affect the yield in the current year, but it decreased the regrowth potential of the crop in the following years, compromising the productivity in the medium-term (Sanderson *et al*., 1999; Monti *et al*., 2008). In case of double harvest systems, it was observed that the phenological stage at which the first cut occurs is not relevant to the switchgrass persistence, however it was observed that the regrowth potential decreased as the first cut was delayed to later stages during the season (Anderson and Matches, 1983). In this study, we did not observe any effect due to the harvest timing and frequency. Indeed, averaging the two-years results, the two switchgrass varieties produced similarly in the six systems. The first cut in the early season in S0-su and S0-c did not produce any negative effect in the final yield, compared to S0. Only S50 reached higher production, in the two years, maybe, as a consequence of mineral N supply. Moreover, in both relay-intercropping systems, the early cut at re-sprouting of the second year, due to the overlapping with the maturity stage of cool-season legumes, did not affect annual yield of the two switchgrass varieties.

The reduction of switchgrass productivity from 2014 and 2015, most likely was caused by the different weather condition and/or by the age of plantations, five years old when the experiment was established. The possible effect of the plantation age complies with the long-term observations of Alexopoulou *et al*. (2015), which showed a significant decreasing of biomass accumulation under Mediterranean condition (Greece), starting from the fifth year, for both lowland and upland varieties.

Nevertheless, in the two years, we observed a different response of the two switchgrass varieties to the treatments. On average, the biomass production of Blackwell differed significantly from 2014 to 2015, while Alamo yielded quite similar in the two years. The yield reduction of Blackwell in 2015 was quite marked in all the evaluated systems. Conversely, in 2015, the yield of Alamo was slightly lower in 5 out of 6 systems, since in Ssu was significantly higher.

The difference between the two varieties could be a consequence of the different growing cycles. Indeed, Alamo was characterized by a longer growing cycle than Blackwell, and the summer harvest was performed with a gap of 39 and 29 days in 2014 and 2015, respectively. Thus, in 2014, Alamo was harvested once less than Blackwell in all the systems, due to delayed flowering, that did not allow the crop regrowth in September. Thus, the different harvest frequency in 2014 could have reduced the re-sprouting in 2015, more in Blackwell than in Alamo.

Conclusions

Relay intercropping by overseeding of cool-season legumes did not decrease switchgrass productivity compared with the pure stands. Sulla, a biennial legume typical of the Mediterranean environments characterized by dry summer and clay soil, demonstrated a good response to overseeding on mature switchgrass stands, allowing a better establishment of the crop during the first year and a positive effect on the re-growth in the second year. This allowed a significant improvement of the total herbage production of the relay-intercropping system and the enhancement of the biomass yield of both switchgrass varieties during the second year, compared with the pure stands fertilized and unfertilized control.

In our study, the use of annual legumes, as crimson clover, showed that the effectiveness of the overseeding practices can be affected by the uncertainty of weather condition, during the germination and seedling emergence stages in late summer and early autumn, as occurred in the second year of our experiment. Nevertheless, the total annual herbage production has been enhanced by the cool-season legume cultivation, indicating the potential of over-seeding with legumes in providing biomass for different switchgrass-based land uses such as feed or combined feed-fuel production. In addition, the early-spring harvest of legumes, can allow to collect high-nutritive forage biomass in the Mediterranean and to reduce the competitions for resources such as light, water and nutrient between switchgrass and the cool-season legume, without affecting the switchgrass productivity compared with the pure stands. However, under Mediterranean conditions, further studies are needed in order to investigate the suitability of legumes intercropping with switchgrass and to evaluate the effect of this system on the overall yield and nutritive value of biomass during the crop cycle as well as to assess the potential benefits for the agroecosystem in the long term. In fact, crimson clover and sulla, could enhance not only yield and nutritive values of total biomass but could lead to several environmental and economic benefits such as reducing soil erosion, improving carbon stock into the soil, reducing the risk of N leaching and minimizing fertilizer costs. Moreover, aiming to optimize synergies between legumes and switchgrass, it could be useful to analyse different harvest frequencies and management options to reduce the competitions (*e.g.* light, water and nutrient) in spring and emphasizing the facilitations (*e.g.* N fixation by legumes, yield increase).

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