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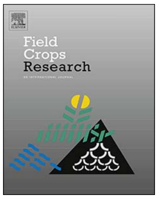

Lignocellulosic biomass production of Mediterranean wild accessions (*Oryzopsis miliacea*, *Cymbopogon hirtus*, *Sorghum halepense* and *Saccharum spontaneum*) in a semi-arid environment

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ABSTRACT

Sustainable biomass production mostly relies on cultivation practices employing low external input supply. Wild germplasm might be suited for low-input techniques while providing enough output. The present study investigated four native Mediterranean perennial grasses (*Oryzopsis miliacea*, *Cymbopogon hirtus*, *Sorghum halepense*, and *Saccharum spontaneum* L. ssp. *aegyptiacum*), with an autumn and winter harvest regime in a 4-year field trial in Sicily (south of Italy). Species, cultivation year and harvest time had significant effects on whole season crop water use efficiency, energy efficiency and biomass quality. Species and cultivation year also significantly affected aboveground biomass yield and net energy yield. The total accumulated harvested yield over the 4-year experiment was the highest in *Saccharum* in both autumn and winter harvests (79.5 and 79.2 Mg DM ha^{-1}), and the lowest in *Cymbopogon* (13.7 and 14.9 Mg DM ha^{-1}). In both harvests, less than 20% of this total biomass was collected for all species at the first year (9.5–11.3% in *Sorghum* and 15.7–18.1% in *Oryzopsis*). Peak biomass was reached at the third (*Saccharum* and *Sorghum*) or at the fourth year (*Orzyzopsis* and *Cymbopogon*) in autumn, and at the third year in winter harvest (all species). Water use efficiency was higher in the autumn than in the winter harvest (2.06 and 1.75 g L^{-1}, respectively), whilst the opposite was observed for energy efficiency (35.7 and 38.6 GJ ha^{-1}, respectively). Biomass structural compounds (hemicellulose, cellulose and acid detergent lignin) were higher in winter than autumn, while protein, lipid and ash contents were higher in autumn than winter.

1. Introduction

Research on perennial grasses for biomass production relies on a limited number of dedicated species suited to the different climatic conditions of Europe. The C_4 *Miscanthus* x *giganteus* and *Panicum virgatum* are high-yielding in temperate environments of northern and central Europe (Lewandowski et al., 2003; Zegada-Lizarazu et al., 2010), while the C_3 *Phalaris arundinacea* and *Arundo donax* are most suited further north and south, respectively (Lewandowski et al., 2003; Mantineo et al., 2009; Cosentino et al., 2014, 2016). Outstanding biomass yields have been obtained with these species when grown in non-limiting conditions; however, when managed in low-input systems (i.e., rainfed, unfertilized, etc.) yields were variable (Alexopoulou et al., 2015). These species are largely undomesticated and research in breeding and agronomic practices optimization is still needed (Zegada-Lizarazu et al., 2010). Nowadays, considerable attention has been paid to the effects of regional climate on plant development in order to identify the optimal genotype for a particular location (Nunn et al., 2017). However, there are considerable numbers of plants still largely unexplored, highly-resource-use-efficient and well-performing in locations with specific constraints.

In the Mediterranean area, for instance, water and heat stress usually limit plant production to different extents (Sánchez et al., 2015). In these conditions, species abundantly widespread in hot, drought- and harsh-prone environments should be investigated. However, the native germplasm of Mediterranean perennial grasses is nearly unexplored for bioenergy production to date (Sulas et al., 2015).

In this sense, Cosentino et al. (2015) proved that *Saccharum spontaneum* spp. *aegyptiacum,* native to northern Africa, is well adapted to the drought conditions of Southern Europe, showing biomass yields even higher than *Miscanthus* x *giganteus* and *Arundo donax* grown in the same experimental area under well-watered and rainfed conditions. In addition, Scordia et al. (2015a) indicated this species as a valuable candidate in environments characterized by drought stress, high

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temperatures and high vapor pressure deficits due mainly to the long green LAI maintenance and CO_2 assimilation, high net increase of biomass production per unit of intercepted light and per unit of transpired water. It would be worth investigating whether other Mediterranean perennial grasses have the typical biomass crop traits. For instance, *Cymbopogon hirtus* and *Oryzopsis miliacea,* which naturally occur in hash environments, are used as forage resources on the south side of the Mediterranean Basin and in South Africa (Foury, 1956; Lloyd and Moore, 2002). *Sorghum halepense* has recently been used to develop perennial sorghum (*S. bicolor*) as a bioenergy crop, although it is considered one of the worst noxious weeds in the USA (Price et al., 2006).

The native germplasm of Mediterranean perennial grasses could therefore represent a potential source of bioenergy crops or a resource for plant breeding and genetics due mainly to traits of resistance and phenotypic plasticity to several biophysical constraints (Tilman et al., 2006). On the other hand, the knowledge of their ecology, biology, physiology and agronomy must be improved before they can be recommended as candidate bioenergy crops.

In addition to the yield, biomass quality is of paramount importance to optimize bioconversion processes. One of the major determinants of biomass productivity (Beale and Long, 1997; Heaton et al., 2009; Hoagland et al., 2013), stand longevity (Heaton et al., 2009; O'Flynn et al., 2014) and quality of perennial grasses (Baxter et al., 2014; Kludze et al., 2013) is harvest time (Monti et al., 2015). Within the same species or genotype, harvest time affects cell wall composition, ash, stem to leaf ratio and biomass water content, in turn conditioning post-harvest logistics and bioconversion pathways (Monti et al., 2008, 2015).

To this end, the present study investigated autochthonous and undomesticated Mediterranean perennial grasses as novel candidate lignocellulosic bioenergy crops for the semi-arid Mediterranean area. *Oryzopsis miliacea*, *Cymbopogon hirtus*, *Sorghum halepense*, and *Saccharum spontaneum* ssp. *aegyptiacum* were compared in a four-year field trial under low-input agronomic practices with an autumn and winter harvest regime.

2. Materials and methods

2.1. Field trial set-up

The field trial was carried out from spring 2010 to winter 2014 at the Experimental farm of the University of Catania, Italy (37°25′ N., 15°03′ E., 10 m a.s.l.). In a randomized block, four species were compared in a split-plot design replicated three times: *Oryzopsis miliacea* (L.) Asch. & Schweinf. (sin. *Piptatherum miliaceum* L. Coss.), *Cymbopogon hirtus* (L.) Janchen (sin. *Hyparrhenia hirta* (L.) Stapf.), *Sorghum halepense* (L.) Pers., and *Saccharum spontaneum* L. ssp. *aegyptiacum* (Willd.) Hackel. The main plots were used for the species, while the sub-plots were used to analyse the harvest time, namely autumn and winter. Each main plot measured 100 m^2 (10 × 10 m), while each sub-plot 50 m^2 (10 × 5m).

Field and bed preparation followed an autumn ploughing and spring disk-harrowing before transplant. Local populations widespread near the experimental field provided enough material for clonal propagation either from rhizome (*Sorghum* and *Saccharum*) or clump division (*Oryzopsis* and *Cymbopogon*).

Clones were manually transplanted at a density of 1.0 plant m^{-2} (1.0 × 1.0 m). At transplant, basic fertilization with 50 kg N ha^{-1} as ammonium sulfate and 50 kg P_2O_5 ha^{-1} as superphosphate was applied. Irrigation was only applied in the first year to ensure plant establishment (150 mm), otherwise the plants were rainfed. Weeds were mechanically controlled during the first year. From the second year after planting, no fertilization, irrigation, weeding or other agronomic inputs were provided.

2.2. Measurements

The main meteorological parameters, such as maximum and minimum temperatures and rainfall, were measured by a weather station connected to a data logger (Delta-T, WS-GP1 Compact) located 150 m from the experimental field. The reference crop evapotranspiration (ET_0) was calculated from the evaporation pan (mm d^{-1}) by the pan coefficient of 0.80 (pan placed in dry fallow area, medium RH, light wind speed, windward side 1 m), as reported by Doorenbos and Pruitt (1977). Data were grouped on a ten-day basis, from sprouting to the end of each growing season.

The soil moisture (mm) was modelled to estimate the drought stress over the growing years. The available soil moisture was calculated as difference between the field capacity (27% of dry soil weight) and the wilting point (11% of dry soil weight) in a soil bulk density of 1.1 g cm^{-3} and a rooting depth of 0.9 m. This was decreased by an availability coefficient of 50% to obtain the maximum plant available water, which was increased by the rainfall and irrigation and decreased by the crop evapotranspiration by using the crop coefficients adopted for *Miscanthus* × *giganteus* and *Arundo donax* grown in the same area (Cosentino et al., 2007, 2014). On days when the soil moisture fell below 20% of the maximum plant available water, the plants were considered to be suffering from drought stress (Nunn et al., 2017).

Harvest took place at the beginning of autumn (end of September 2010, 2011, 2012 and 2013) and in winter-time (mid February 2011, 2012, 2013 and 2014).

At harvest, edge plants were removed in each plot to weight the biomass within 12 m^2. Dry biomass yield was calculated by weighing sub-samples of fresh biomass and after oven drying it at 65 °C until constant weight.

The whole season crop water use efficiency (g L^{-1}) was calculated as the ratio between dry biomass yield and crop water use (CWU) from spring-regrowth up to harvest, in both winter and autumn growing seasons. The CWU was calculated according to Cosentino et al. (2014) taking into account the irrigation at establishment (I), the rainfall (R) and the difference between soil water content at 0–90 cm soil depth between a first and a second measurement (ΔC). Soil water content was measured gravimetrically, collecting soil samples in three replicates per plot and oven drying at 105 °C until constant weight.

2.3. Biomass quality

Oven-dried samples (whole aboveground biomass) collected at the autumn or winter harvest were ground through a 1-mm screen in an IKA mill (IKA-WERFE, Gmbh & Co., KG, Staufenim Breisgau, Germany). Cellulose, hemicellulose, acid detergent lignin (ADL), proteins, lipids and ash were determined by a near-infrared spectrometer (NIR, SpectraStar™ 2500XL-R, Unity Scientific) provided with a tungsten halogen lamp as light source and a high performance ultra-cooled InGaAs extended range detector. Samples were placed in small powder cups and scanned in duplicate in diffuse reflection measurement mode, wavelength range of 680–2500 nm and accuracy < 0.1 nm. A previous calibration developed by the Ucal complete chemometric calibration software (InfoStar 3.11.0 version) was adopted. The calibration consisted of a regression that correlates spectra and analytic determinations of 240 different lignocellulosic raw materials of *Arundo donax* clones and *Miscanthus* species (stems, leaves or the whole biomass) grown under different agronomic practices and growing seasons. Following a first scan run, spectra of *Oryzopsis, Cymbopogon, Sorghum* and *Saccharum* were also used for further calibration development in the Ucal software. The same biomass samples were analytically determined in triplicate according to the Van Soest et al. (1991) method for structural carbohydrate and ADL by using a raw fiber extractor (FIWE 6, VELP Scientifica Srl, Usmate, Italy), the Kjeldahl method for proteins (Distillation unit B-324, Büchi Italia Srl), the Soxtec-Tecator extraction for lipids (FOSS Analytical, 15 Höganäs, Sweden) and the

ASTM E1755-01 standard for ash.

The statistical methods used in the calibration, for both quantitative and qualitative analysis are the multiple linear regression of derivative absorbance method and multivariate Partial Least Squares (PLS). The PLS is a technique that decomposes the spectrum in a quantitative way by exploiting the correlation between the spectra data and the constituent concentrations. Before developing the final calibration, various pretreatments were applied, such as the cross validation groups (CV Groups), set at 4, the outlier limits, namely the t-student and the global distance, set at 2.5 and 5.0, respectively, and the expansion multiplier, set at 3.

At this point, the software processes a new regression that correlates spectra and analytic determinations. The predictive ability of the calibration equations was assessed by the standard error and the coefficient of determination in calibration (SEC and R2C, respectively), and the standard error and the coefficient of determination in cross-validation (SECV and R2CV, respectively).

As both SEC and R2C parameters do not take the variance of the regression coefficients into account, the SECV and R2CV were used to indicate the average prediction error and the proportion of the variance of the dependent variable explained by the regression. These parameters indicate the efficiency of the predictive model and provide an idea of its accuracy.

In addition, the residual predictive deviation (RPD), i.e., the ratio of the standard deviation of the analyzed character and the standard error of the cross-validation, was also taken into account to estimate the efficiency of the calibration.

The new calibration was then used to obtain model robustness from NIRs for cellulose, hemicellulose, ADL, proteins, lipids and ash of *Oryzopsis, Cymbopogon, Sorghum* and *Saccharum*.

2.4. Energy balance

Energetic costs included energy inputs of materials, technical means and farming practices to establish, manage and harvest perennial grasses. Energy inputs were evaluated in two separate phases, namely establishment (first year management) and post-establishment (after the first harvest) in both harvest regimes.

Both direct and indirect energy costs of mechanization, irrigation, fertilizers, and propagation materials were as in Mantineo et al. (2009). From the second growing season, no inputs were provided and only energy costs of harvesting were considered. An overview of the energy input applied at establishment and post-establishment phases is shown in Table 1.

Outputs were determined as biomass composition in terms of carbohydrates, proteins and lipids by using energy conversion factors proposed by Odum (1988) for food: 0.0167 MJ DM g^{-1} for carbohydrates; 0.0209 MJ DM g^{-1} for proteins and 0.0385 MJ DM g^{-1} for lipids. The carbohydrate energy conversion factor was also applied for lignin (ADL). The low heating value (MJ kg^{-1}) of each crop was obtained by multiplying the conversion factor by the amount of each compound in 1 kg of dry biomass. The net energy yield (EY, GJ ha^{-1}) and the energy efficiency (EE, GJ ha^{-1}) at the farm gate were calculated according to Mantineo et al. (2009). It is worth noting that the present study focused on the impacts of cultivation practices only, thus the complete life cycle assessment of biomass conversion, use-phase and end of life of the bio-system in comparison with the conventional reference system was not attempted.

Table 1
Energy input (GJ ha^{-1}) for establishment and first year management phase, and post-establishment management phase of native perennial grasses.

Management	Establishment	Post-establishment
Soil tillage	2.58	–
Transplanting	5.80	–
Fertilization	8.67	–
Weeding	0.70	–
Irrigation	25.53	–
Harvest	2.94	2.94

2.5. Statistical analysis

Biomass DM yield, WUE, biomass composition, EY and EE were subjected to the two-way analysis of variance (ANOVA) using repeated measurements in time (SPSS, PASW Statistics 18). Measurements were performed throughout years within subjects, while species and harvest time as between subjects. Percentage data were arcsine $\sqrt{\%}$ transformed before the analysis. Duncan's post-hoc test was used for mean separation at 95% confidence level.

3. Results

3.1. Meteorological data and available soil water content

Air temperatures throughout growing seasons reflected the typical trend of the southern Mediterranean environment (Fig. 1). Maximum temperatures were recorded from June to August, between 28.4 and 36.8 °C, and minimum temperatures in January-February, between 2.0 and 3.1 °C. In winter growing seasons (from February to February), yearly mean temperature ranged from 17.5 °C (2011/12) to 18.3 °C (2013/14), averaging 17.8 °C across the four-year experiment. In autumn growing seasons (from September to September), yearly mean temperature ranged from 17.7 °C (2011/12) to 20.2 °C (from establishment to September 2010), averaging 18.4 °C.

Rainfall amount changed considerably throughout growing seasons. In the winter regime, it ranged between 748.3 mm (2011/12) and 390.1 mm (2013/14). In the autumn regime, it ranged from 689.9 mm (2011/12) to 312.2 mm (2012/13), averaging 421.6 mm and 548.2 mm in autumn and winter, respectively. In both regimes, rainfall distribution was quite favorable from spring to autumn (from equinox to equinox) 2011/12 and 2012/13 (325.4 and 291.6 mm, respectively); this period covers crop sprouting, stem elongation and beginning of canopy senescence in this area. On the other hand, whole season ET_0 in the winter regime was between 921.8 mm in 2011/12 and 1022.8 mm in 2012/13. In the autumn regime, ET_0 ranged from 760.9 mm (2010/11) to 1009.5 mm (2012/13), averaging 920.3 mm and 980.1 mm in autumn and winter, respectively.

May to September represents the period with the highest ET_0 in this area (3.8-4.1 mm day^{-1}). Rainfall in those months was between 158.6 mm in 2011/12 and only 36.8 mm in 2012/13.

The soil moisture in the first 90 cm depth was non-limiting throughout the first growing season due to 150 mm of irrigation supplied (Fig. 2). From the second year, irrigation was not provided and soil moisture exceeded the 20% maximum plant available water from late May to the end of summertime, except when significant rainfall events occurred. The modelled soil moisture suggests that rainfed crops were not water stressed from mid-September through mid-May; on the contrary, they were subjected to severe water stress through most of the summer season in this environment.

3.2. Biomass yield (ABY) and water use efficiency (WUE)

Cultivation year and species had significant effects ($p \leq 0.05$) on aboveground dry biomass yield (ABY), while harvest time did not. Significant interactions (except harvest x species) were found. The main effects, including harvest time, and interactions were also significant on whole season WUE (Table 2).

As expected, ABY was the lowest at the first harvest in all species and at all harvest times. *Saccharum* showed a sharp ABY increase from the second cultivation year in both harvest times, while the other species showed a consistent increase from the third year (Fig. 3). In the

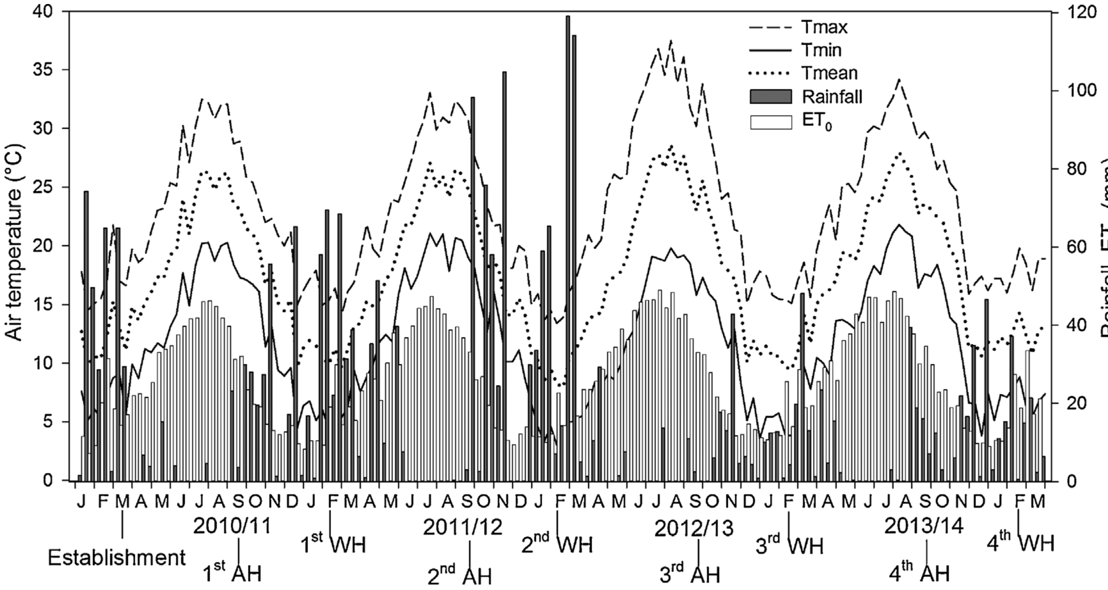

Fig. 1. Meteorological trend [maximum, mean and minimum air temperatures (°C), rainfall and reference evapotranspiration (mm)] at the Experimental Field of the University of Catania (10 m a.s.l., 37° 24′ N, 15° 03′ E) from 2010/11 to 2013/2014 autumn (AH) and winter (WH) harvest times.

winter growing season, the ABY peak was reached at the third harvest in all species: 30.8 Mg DM ha^{-1} in *Saccharum*, 13.8 in Sorghum, 9.4 in *Oryzopsis* and 5.6 in *Cymbopogon.* Yield decreases followed in all species at the fourth harvest.

In the autumn growing season, the ABY peak was reached at the third harvest in *Saccharum* and *Sorghum* (27.7 and 8.1 Mg DM ha^{-1}, respectively) followed by a less consistent ABY decline at the fourth harvest as compared with the winter regime. On the other hand, *Oryzopsis* and *Cymbopogon* steadily increased, reaching a peak at the fourth harvest (5.8 and 4.9 Mg DM ha^{-1}, respectively).

Across harvest times and cultivation years, *Saccharum* was the highest yielding species (19.8 Mg DM ha^{-1}) followed by *Sorghum* (6.6 Mg DM ha^{-1}). *Oryzopsis* and *Cymbopogon* showed the lowest and not statistically different ABY (4.5 and 3.6 Mg DM ha^{-1}, respectively). Across cultivation years and species, the winter season was slightly higher, but not statistically different than the autumn one (8.9 and 8.2 Mg DM ha^{-1}, respectively).

The water used by the crop (CWU) varied between growing seasons (Table 3). The average CWU was lower in the autumn than the winter growing season (460.4 and 618.9 mm). In autumn, CWU ranged between 691.4 mm at the second and 313.4 mm at the fourth cultivation year. In winter, CWU ranged between 795.4 mm at the second and 357.3 mm at the fourth cultivation year.

Table 2
Repeated measure ANOVA for main effects and interactions on aboveground biomass dry matter yield (ABY, Mg DM ha^{-1}) and water use efficiency (WUE, g L^{-1}) of native perennial grasses at two harvest times (autumn and winter harvest) throughout the four growing seasons (2010/11, 2011/12, 2012/13 and 2013/14).

		ABY		WUE	
Source	df	F-value	P-value	F-value	P-value
Year (Y)	3	353.5	0.000	3799.1	0.000
Harvest (H)	1	1.82	0.195	8.6	0.010
Species (S)	3	219.8	0.000	291.1	0.000
Y x H	3	64.1	0.000	183.6	0.000
Y x S	9	101.0	0.000	995.1	0.000
H x S	3	0.52	0.678	7.3	0.003
Y x H x S	9	4.57	0.017	55.3	0.000

The ratio of ABY over CWU represents the crop water use efficiency (WUE). Across species and cultivation years, the WUE was significantly higher in autumn (2.06 g L^{-1}) than in winter regimes (1.75 g L^{-1}). Across cultivation years and harvest regimes, WUE was the highest in *Saccharum* (4.51 g L^{-1}) and the lowest in *Oryzopsis* and *Cymbopogon* (0.94 and 0.76 g L^{-1}, respectively) (Fig. 4). The third year of winter

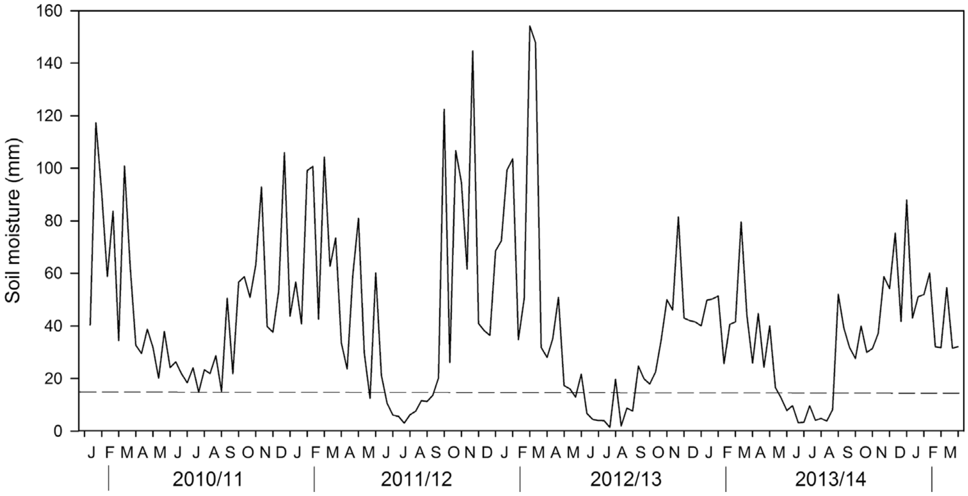

Fig. 2. Modelled soil moisture (mm) through the growing seasons (2010/11, 2011/12, 2012/13 and 2013/14) at the Experimental Field of the University of Catania (10 m a.s.l., 37°25′N lat., 15°03′E long.). The dashed line shows 20% maximum plant available water below which the crop is considered to be water stressed.

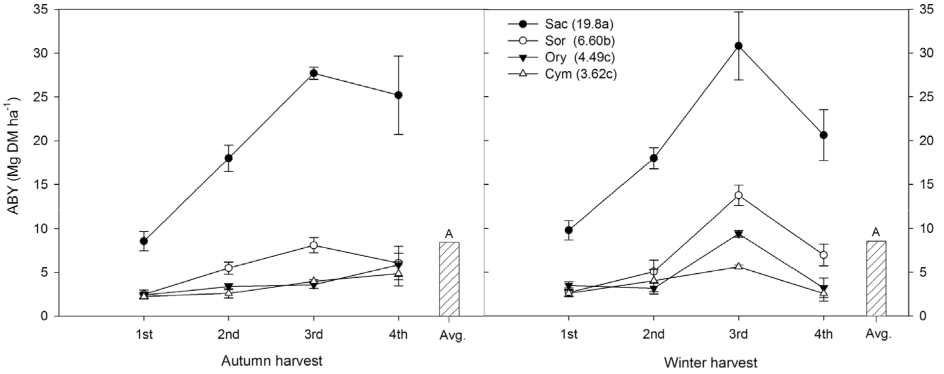

Fig. 3. Aboveground biomass dry matter yield (ABY, Mg DM ha^{-1}) in four consecutive winter and autumn growing seasons of native perennial grasses: *Oryzopsis miliacea*, *Cymbopogon hirtus*, *Sorghum halepense* and *Saccharum spontaneum* ssp. *aegyptiacum*. Different capital letters represent statistical significance of harvest time main effect ($p \leq 0.05$); different small letters represent statistical significance of species main effect ($p \leq 0.05$).

Table 3
Crop water use (CWU, mm) of native Mediterranean perennial grasses in subsequent autumn and winter growing seasons.

Growing season	Rainfall	Irrigation	ΔC	CWU
	(mm)	(mm)	(mm)	(mm)
1st Autumn	335.2	150.0	1.1	486.3
2nd Autumn	689.9	0.0	1.5	691.4
3rd Autumn	349.3	0.0	1.4	350.6
4th Autumn	312.2	0.0	1.2	313.4
Avg. autumn	*421.6*	–	*1.3*	*460.4*
1st Winter	636.6	150.0	4.6	791.2
2nd Winter	748.3	0.0	47.1	795.4
3rd Winter	417.8	0.0	53.8	471.6
4th Winter	390.1	0.0	−32.8	357.3
Avg. winter	*548.2*	–	*18.2*	*603.9*

regime led to the highest WUE in all species, whilst *Oryzopsis* and *Cymbopogon* attained the highest WUE at the fourth year in the autumn regime.

3.3. Biomass quality

Cultivation year, species and harvest time had significant effects ($p \leq 0.05$) on hemicellulose, cellulose, acid detergent lignin (ADL), protein, lipid and ash content. Significant interactions were also detected (Table 4).

Across cultivation years and species, structural compounds were higher in winter than in autumn regimes [(i.e., hemicellulose (33.9 vs 32.7%), cellulose (36.5 vs 34.7%) and ADL (8.1 vs 7.4%)]. On the contrary, autumn showed higher non-structural compounds and ash content than winter [proteins (5.0 vs 4.3%), lipids (1.9 vs 1.3%) and ash (6.1 vs 5.4%)] (Fig. 5).

Across cultivation years and harvest regimes, *Oryzopsis* showed the highest protein (8.0%), lipid (1.7%), ADL and ash (9.7 and 5.8%, respectively), and the lowest hemicellulose and cellulose content (31.9 and 30.7%, respectively). *Sorghum* showed the highest hemicellulose and cellulose content among species (35.5 and 39.6%, respectively) but the lowest protein and lipid content (2.9 and 1.3%, respectively). Ash content in *Saccharum* was as high as in *Oryzopsis* (5.8%), while ADL was the lowest (7.1%). The other constituents were at the middle range as in *Cymbopogon*.

Across species and harvest times, hemicellulose, protein, lipid and ash contents were higher at the first harvest, and steadily decreased in the remaining cultivation years. On the contrary, cellulose and ADL steadily increased from the first up to the fourth year.

Structural compounds showed low coefficient of variabilities (CV) as compared with non-structural compounds across cultivation years and species in both harvest times. In the winter regime, CV of hemicellulose, cellulose, ADL and ash content was lower than 20% (i.e., from 6.2% to 18.7%), while protein and lipid content were higher (44.9% and 26.7%, respectively). In the autumn regime, CV was 4.8% for hemicellulose, 7.6% for cellulose, 20.1% for ADL and 15.5% for ash. Again, protein and lipid showed the highest CVs (59.7% and 21.4%, respectively).

3.4. Energy balance

Energy costs computed to establish native perennial grasses included both direct and indirect costs of soil ploughing and harrowing, fertilization, transplant of clonal material, irrigation, weed control and harvesting, adding up to 46.22 GJ ha^{-1} in both harvest regimes. Post-establishment energy costs referred only to harvest, accounting for 2.94 GJ ha^{-1} (Table 1).

Calculated low heating value (MJ kg^{-1}) across cultivation years showed low CVs within species in both harvest regimes. In winter, *Saccharum* averaged 15.1 (CV of 3.6%), *Sorghum* 16.2 (CV of 2.7%), *Oryzopsis* 14.5 (CV of 3.0%) and *Cymbopogon* 15.5 (CV of 2.7%). In autumn, *Saccharum* averaged 15.5 (CV of 0.6%), *Sorghum* and *Oryzopsis* 15.4 (CV of 3.5% and 4.2%, respectively), and *Cymbopogon* 15.9 (CV of 2.8%). Significant effects of cultivation year and species ($p \leq 0.05$) were found on net energy yield (EY), while harvest time had no effect.

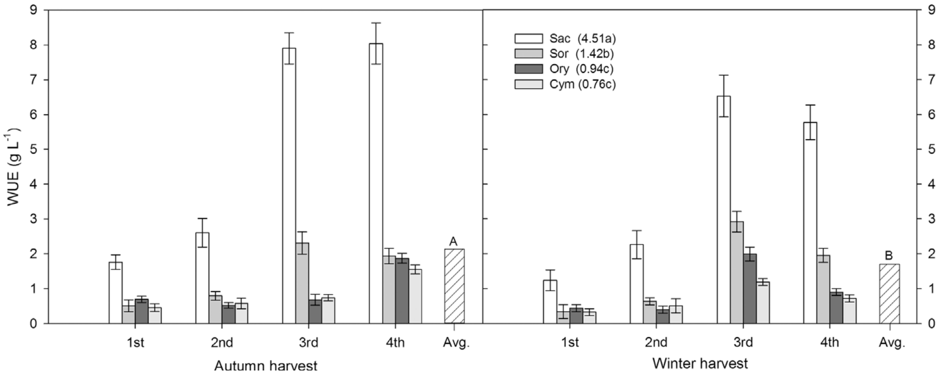

Fig. 4. Water use efficiency (WUE, g L^{-1}) in four consecutive winter and autumn growing seasons of native perennial grasses: *Oryzopsis miliacea*, *Cymbopogon hirtus*, *Sorghum halepense* and *Saccharum spontaneum* ssp. *aegyptiacum*. Different capital letters represent statistical significance of harvest time main effect ($p \leq 0.05$); different small letters represent statistical significance of species main effect ($p \leq 0.05$).

Table 4
Repeated measure ANOVA for main effects and interactions on biomass composition (% w/w) of native perennial grasses at two harvest times (autumn and winter harvest) throughout the four growing seasons (2010/11, 2011/12, 2012/13 and 2013/14). *Acid detergent lignin.

		Hemicellulose		Cellulose		ADL*		Protein		Lipid		Ash	
Source	df	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value	F-value	P-value
Year (Y)	3	315.1	0.000	447.5	0.000	66.1	0.000	2008.6	0.000	808.6	0.000	110.7	0.000
Harvest (H)	1	325.1	0.000	15.37	0.001	43.4	0.000	693.1	0.000	1399.2	0.000	128.1	0.000
Species (S)	3	361.0	0.000	3527.3	0.000	208.2	0.000	8678.4	0.000	158.4	0.000	21.4	0.000
Y x H	3	43.2	0.000	9.86	0.003	6.22	0.024	109.8	0.000	19.3	0.000	30.1	0.000
Y x S	9	69.1	0.000	116.9	0.000	5.18	0.011	198.6	0.000	178.1	0.000	47.3	0.000
H x S	3	316.7	0.000	1248.1	0.000	68.9	0.000	1401.0	0.000	48.8	0.000	47.9	0.000
Y x H x S	9	60.1	0.000	201.6	0.000	4.50	0.018	79.2	0.000	18.6	0.000	82.6	0.000

Interactions were also significant (except harvest x species). The main effects and interactions regarding energy efficiency (EE) were all significant (Table 5).

Across cultivation years and harvest regimes, *Saccharum* EY was significantly higher than *Sorghum* (277.5 and 88.4 GJ ha^{-1}), which in turn was higher than both *Oryzopsis* and *Cymbopogon* (49.2 and 40.3 GJ ha^{-1}, respectively). Across species and cultivation years, harvest regime did not show significant differences and attained 115.3 GJ ha^{-1} in winter and 106.1 GJ ha^{-1} in autumn (Fig. 6). As expected, the first harvest led to the lowest EY in all species, with negative values in

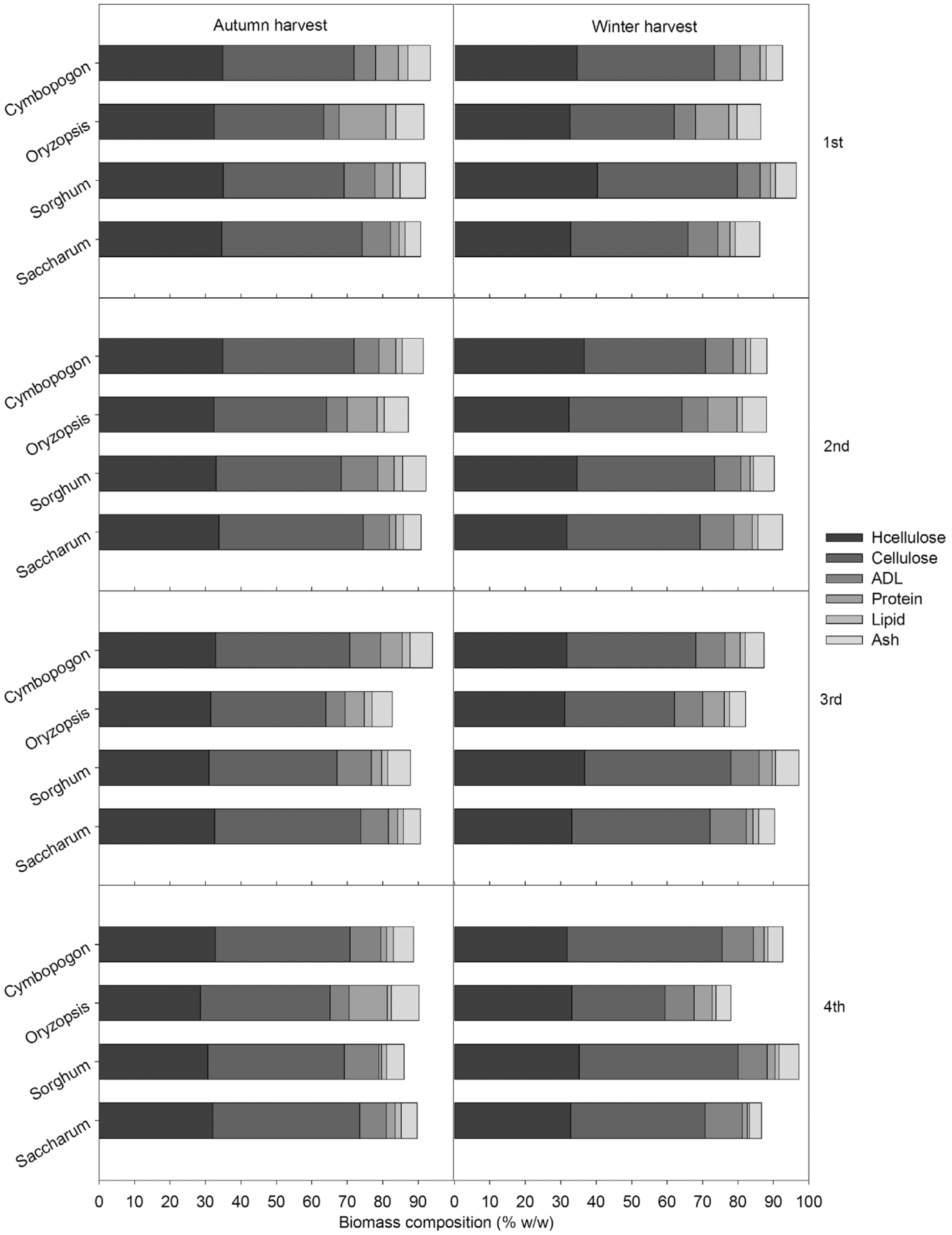

Fig. 5. Raw material composition (% w/w) in four consecutive winter and autumn growing seasons (2010/11, 2011/12, 2012/13 and 2013/14) of native perennial grasses: *Oryzopsis miliacea*, *Cymbopogon hirtus*, *Sorghum halepense* and *Saccharum spontaneum* ssp. *aegyptiacum*.

Table 5
Repeated measure ANOVA for main effects and interactions on net energy yield (EY, GJ ha^{-1}) and energy efficiency (EE, GJ ha^{-1}) of native perennial grasses at two harvest times (autumn and winter harvest) throughout the four growing seasons (2010/11, 2011/12, 2012/13 and 2013/14).

		EY		EE	
Source	df	F-value	P-value	F-value	P-value
Year (Y)	3	894.5	0.000	24953.5	0.000
Harvest (H)	1	4.21	0.057	37.2	0.000
Species (S)	3	484.6	0.000	3568.2	0.000
Y x H	3	130.9	0.000	1936.4	0.000
Y x S	9	204.2	0.000	5544.6	0.000
H x S	3	4.10	0.240	26.8	0.000
Y x H x S	9	7.37	0.000	149.8	0.000

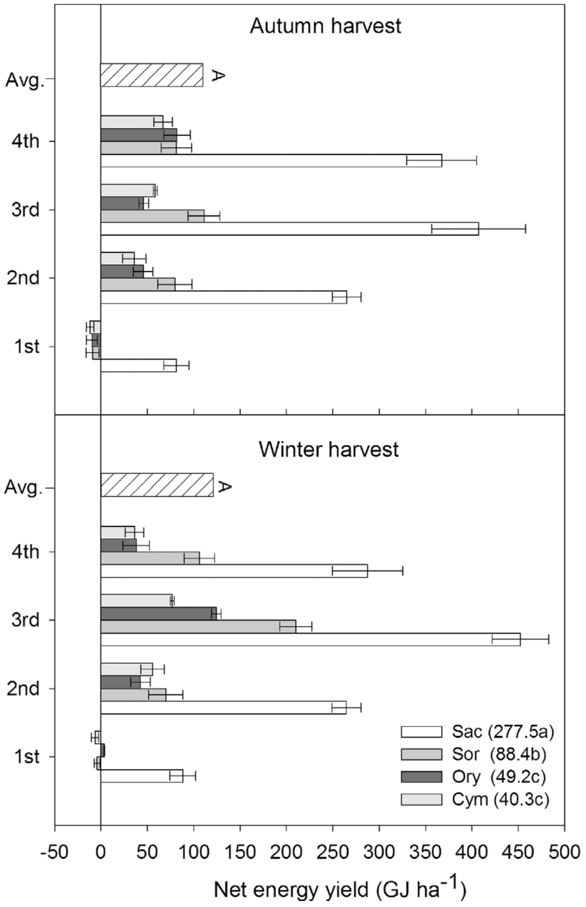

Fig. 6. Net energy yield (EY, GJ ha^{-1}) in four consecutive winter and autumn growing seasons of native perennial grasses: *Oryzopsis miliacea*, *Cymbopogon hirtus*, *Sorghum halepense* and *Saccharum spontaneum* ssp. *aegyptiacum*. Different capital letters represent statistical significance of harvest time main effect ($p \leq 0.05$); different small letters represent statistical significance of species main effect ($p \leq 0.05$).

Sorghum, *Cymbopogon* and *Oryzopsis* in the autumn regime (-9.2, −11.8 and −9.8 GJ ha^{-1}, respectively). *Sorghum* and *Cymbopogon* showed negative values also at the first harvest in winter regime (-4.2 and −6.4 GJ ha^{-1}, respectively).

The EY steadily increased from the second year to reach a peak at the third year in the winter regime in all species (from 452.3 GJ ha^{-1} in *Saccharum* to 76.7 GJ ha^{-1} in *Cymbopogon*). In the autumn regime, *Saccharum* and *Sorghum* showed the EY peak at the third year as well (407.1 and 107.1 GJ ha^{-1}, respectively), while *Oryzopsis* and *Cymbopogon* peaked at the fourth year (81.9 and 66.8 GJ ha^{-1}, respectively).

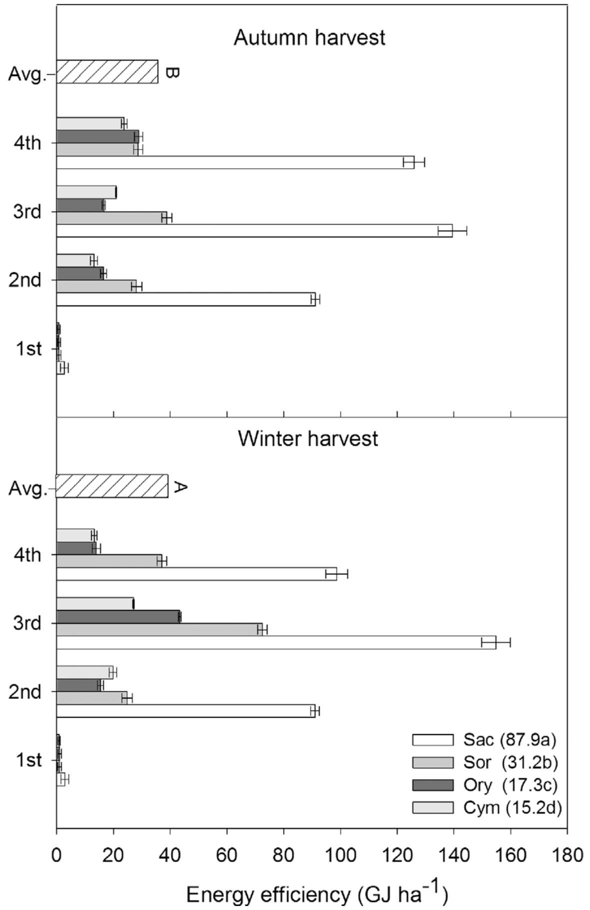

Fig. 7. Energy efficiency (EE, GJ ha^{-1}) in four consecutive winter and autumn growing seasons of native perennial grasses: *Oryzopsis miliacea*, *Cymbopogon hirtus*, *Sorghum halepense* and *Saccharum spontaneum* ssp. *aegyptiacum*. Different capital letters represent statistical significance of harvest time main effect ($p \leq 0.05$); different small letters represent statistical significance of species main effect ($p \leq 0.05$).

The energy efficiency (EE) across cultivation years and harvest regimes was significantly higher in *Saccharum* than *Sorghum* (87.9 and 31.2 GJ ha^{-1}), *Oryzopsis* and *Cymbopogon* (17.3 and 15.2 GJ ha^{-1}). Across species and cultivation years, EE was significantly higher in winter than in autumn (38.6 and 35.7 GJ ha^{-1}), as shown in Fig. 7. In this case too, the first harvest led to the lowest EE, although values were positive in all species. The EE peak was reached at the third harvest in all species in the winter (from 154.8 GJ ha^{-1} in *Saccharum* to 27.1 GJ ha^{-1} in *Cymbopogon*) and at the third (*Saccharum* – 139.4 GJ ha^{-1} and *Sorghum* − 38.8 GJ ha^{-1}) or at the fourth year (*Oryzopsis* – 28.8 GJ ha^{-1} and *Cymbopogon* − 23.7 GJ ha^{-1}) in the autumn regime.

4. Discussions

The aim of the present study was to compare four native Mediterranean perennial grasses as candidate lignocellulosic bioenergy crops under low-input agronomic practices in an early or late harvesting. Biomass yield, whole season water use efficiency, biomass quality, energy yield and efficiency were determined over a four-year period in a south Mediterranean environment dominated by drought stress and uneven rainfall distribution during the greatest part of the vegetative crop development.

4.1. Aboveground biomass yield (ABY) and water use efficiency (WUE)

All species showed the lowest ABY and WUE at the first harvest, which is a recognized drawback for perennial grasses (Scordia et al., 2015b). Steady yield increases were observed from the second year as shown by the significant cultivation year and the significant interaction of main effects.

Despite the low plant density at transplant (1 plant m^{-2}), the two rhizomatous species - *Saccharum* and *Sorghum* – were able to cover nearly all the ground area at the second year, increasing the biomass yield almost two-fold as compared with the previous year in both harvest regimes. Biomass production peaked at the third year in the winter season, 2.9-fold in *Oryzopsis*, 2.7-fold in *Sorghum*, 1.7-fold in *Saccharum* and 1.3-fold in *Cymbopogon* as compared with the previous growing season. At the fourth year, all species showed a general decline.

In the autumn season, *Saccharum* and *Sorghum* showed a similar trend to the winter season, but the yield decline at the fourth year was less consistent. On the other hand, *Oryzopsis* and *Cymbopogon* gradually increased from the first up to the fourth year (2.4 and 2.1-fold from the first to the peak).

Alexopoulou et al. (2015) suggested that biomass yield is mostly driven by rainfall amount and distribution when perennial grasses are rainfed in the Mediterranean environment.

At the first and second winter growing seasons, rainfall was higher than the four-year mean (548.2 mm), accounting for 636 and 748 mm, respectively. On the contrary, at the third and fourth year annual rainfall was lower, 417 and 390 mm, respectively. Surprisingly, biomass yield peaked at the third growing season. Aside from the physiological behavior of perennial grasses, which are used to peak ABY from the second/third growing season (Cosentino et al., 2006; Angelini et al., 2009; Alexopoulou et al., 2015), rainfall distribution played a key role in the third year as 292 mm were recorded between March and October. This period covers crop sprouting, stem elongation and beginning of canopy senescence of perennial grasses in the Mediterranean area. In addition, 338 mm were recorded in January-March of the previous season, which favored soil water restoration for shoot regrowth and mid-seasons growth in spring.

The high ABY achieved at the third year being supported by a favorable distribution rather than high rainfall amount resulted in the overall highest WUE for the winter season. The fourth growing season was quite dry; although 200 mm fell in the period March-October, only 5.9 mm were recorded in summertime leading to extended drought stress during the greatest part of the vegetative crop development. These conditions triggered early crop senescence and increased overwinter biomass losses. In these dry conditions, *Oryzopsis* showed the highest ABY and WUE reduction, and *Saccharum* the lowest as compared with the peak yield.

Rainfall distributions in autumn growing seasons during crop development were the same as in winter ones. However, as crops were harvested at nearly the beginning of senescence, overwinter biomass losses were not detected and WUE improved as observed at the fourth year.

Taken together, it is possible to highlight that rainfall amount is an important resource for biomass production when perennial grasses are grown in rainfed conditions; nonetheless, its distribution throughout the growing season is the main determinant of yield in post-established perennial grasses.

Outstanding ABY and WUE performances of *Saccharum* parallels our previous findings with the winter harvest (Cosentino et al., 2015; Scordia et al., 2015a). Surprisingly, the autumn harvest made it possible to improve the WUE in the physiologically mature *Saccharum* stand. WUE in *Saccharum* was higher than *Miscanthus* x *giganteus* (3.90-5.14 g L^{-1}) and similar to that of *Arundo donax* (5.04-7.63 g L^{-1}) when both crops were rainfed in southern Europe (Mantineo et al., 2009). It is worth noting, however, that methodological approaches could play a crucial role in WUE evaluation and thus in comparison with other studies (Triana et al., 2014). For instance, using the cumulative crop evapotranspiration approach rather than the crop water use approach (as in this work) would decrease the WUE in all species and harvest regimes by 25–30%.

In favorable climatic conditions (third winter growing season), *Sorghum* produced similar biomass yield reported under subtropical areas, between 14 and 17 Mg ha^{-1} (Lewandowski et al., 2003), and a WUE comparable to that of giant reed (Cosentino et al., 2014). Ball et al. (2007) reported that *S. halepense* can routinely produce between 4.5 and 11.2 Mg of hay per ha over an entire growing season in southern USA, which is in line with our findings. However, the unstable yield level of this species suggests that further studies on persistence and testing in multiple locations are needed before recommending it as bioenergy crop. According to the ABY and WUE levels, *Oryzopsis* and *Cymbopogon* cannot be recommended either. As mentioned above, these species were not able to cover the ground area like the rhizomatous ones; thus, the adopted plant density was probably a limiting factor for biomass production. Field trials to optimize plant density could reveal the real potentiality of these grasses. In this regard, Sulas et al. (2015) performed a comparison of several native Mediterranean populations of *Oryzopsis miliacea* established at a plant density of 0.5 × 0.5 m showing great potentialities of this species under the climatic conditions of the island of Sardinia.

In summary, this work investigated whether perennial grasses other than the most financially supported *Miscanthus*, *Arundo* and *Panicum* have the typical biomass crop traits needed for the semi-arid Mediterranean area. *Saccharum* outperformed the other species and can be considered a bioenergy crop option for drought-prone environments. However, a tradeoff is the inability to produce viable seed resulting in restricted genetic diversity for breeding programs associated with the high establishment costs and low multiplication rates. Other clonal propagation options could be explored, such as for example stem cutting, which has been demonstrated to make the multiplication rate several orders of magnitude greater than rhizome propagation for both *Miscanthus* and *Arundo* (Scordia et al., 2015b) although drawbacks of clone plants still remain. Clifton-Brown et al. (2017) argued that breeding of a clonal material is not an easy task; it includes the collection of wild germplasm from native environments to increase genetic diversity and testing in multiple locations to span a diversity of climates and to identify key phenotypic traits and parental combinations. About one decade was required to overcome the technical barriers of using *Miscanthus* as a candidate low-carbon feedstock in the European bioeconomy (Clifton-Brown et al., 2017).

Although *Sorghum*, *Oryzopsis* and *Cymbopogon* are able to produce viable seed in this environment, questions remain on the seed biology and physiology, seedbed preparation methods, sowing time, seedling density and weed control. A great deal must be still done to exploit these species at farm-scale level and to deliver ideotype varieties tailored to the different European environmental conditions.

4.2. Biomass quality

Lignocellulosic biomass is the most abundant and low-cost raw material on earth suitable to develop a competitive, resource-efficient and low-carbon economy in Europe (Scarlat et al., 2015). In accordance with other lignocellulosic feedstock, the chemical composition of *Saccharum*, *Sorghum*, *Oryzopsis* and *Cymbopogon* was primarily made up of structural polysaccharides (cellulose and hemicelluloses), lignin and small fractions of non-structural components, such as protein, lipids, and ash (Scordia et al., 2014).

Although cultivation year, species and harvest main effects differed significantly, structural carbohydrates and acid detergent lignin (ADL) varied little as compared with non-structural compounds. This is of utmost importance in a lignocellulosic bioenergy chain since a stable biomass composition delivered at the bioconversion site avoids continual modifications to processing operations (Arundale et al., 2015).

Basically, non-structural compounds and ash were higher in autumn than winter harvests, whilst structural compounds were higher in winter than autumn. This is in agreement with the physiology of perennial grasses, as nutrients (and thus ash and minerals in the harvestable biomass) are mobilized upwards (from below to aboveground) during the vegetative development and downwards (from above to belowground) after the onset of senescence. It has been demonstrated that flowering and senescence (late harvest) in sixteen *Miscanthus* genotypes (including *M.* x *giganteus*, *M. sinensis* and *M. sacchariflorus*) tended to decrease P, K, Cl, N, Si, ash and moisture contents as compared with early harvest or no-flowering and late-senescing genotypes (Jensen et al., 2016). In perennial grasses, seasonal dynamics of nutrient accumulation and partitioning has been indicated as the main determinant of biomass quality for thermal conversions since lower moisture, ash and inorganic elements avoids slagging, fouling and corrosion of the combustion equipment (Monti et al., 2008; Kludze et al., 2013). Furthermore, too early harvesting might impact on stand longevity, as nutrients are removed in the whole bulk collected (Strullu et al., 2011).

Among species, *Sorghum* showed the highest hemicellulose and cellulose followed by *Saccharum* and *Cymbopogon*, thus with positive traits for second-generation bioethanol production as structural polysaccharides are positively related to the theoretical ethanol yield (Scordia et al., 2014). Recently, Scordia et al. (2010) showed the suitability of *Saccharum* biomass in oxalic acid pretreatment and both hemicellulose and cellulose-derived sugar fermentation to ethanol. On the other hand, the severe slagging propensity of *Saccharum* biomass due to the low ash deformation temperatures indicates that care should be taken with thermochemical conversions operating at high temperatures (Scordia et al., 2016).

Oryzopsis showed the lowest hemicellulose and cellulose content and the highest ADL and ash content, suggesting unsuitable feedstock for both biochemical and thermochemical conversions. Nonetheless, the highest protein and lipid associated with the lowest fiber content indicates the availability of medium-quality forage suitable to optimize forage production and palatability.

In general, cellulose and hemicellulose were in the range reported for other monocot species, agricultural residues and woody crops (Scordia et al., 2014). However, lignin content was generally lower likely due in part to different analytical procedures. For instance, Allison et al. (2012) showed that the method for Klason lignin determination provided much higher values of lignin than measurement as ADL, although data from both methods showed similar trends. Thus, within the limit of this study, the method employed to determine cell wall composition was consistent to compare these species over cultivation years and harvest times.

The ash content of the four species was similar to *Arundo donax* biomass but higher as compared with *M.* x *giganteus* or *M. sinensis* grown in the same area (Scordia et al., 2016).

The higher proportion of ash content in the first years of growth might be explained by the higher incidence of leaves in the whole bulk collected, as leaves are richer in ash than stems (Monti et al., 2008). Similarly, proteins were higher at the first harvest, most likely due to a combination of nitrogen fertilization and higher leaf proportion, as leaves are also richer in N than stems (Monti et al., 2008).

Overall, cellulose and lignin increased and hemicellulose showed small but significant decreases in the growth years following establishment, which is in agreement with a long-term study on *Phalaris acquatica* (Pappas et al., 2014). The loss of hemicellulose in mature stands has been associated with the increased deposition of cellulose and lignin, or the replacement of hemicellulose in the cell matrix as by lignin (Allison et al., 2012).

4.3. Energy balance

The energy balance was calculated to compare the net energy yield (EY) and the energy efficiency (EE) at the farm gate of the four native perennial grasses at establishment (first cultivation year) and post-establishment (from second to fourth cultivation year) in two harvest regimes. Energy input and output, and in turn EY and EE, differed among species and cultivation phase, and varied according to biomass production achieved and external input used.

As expected, the low biomass yield at establishment, accompanied by high energy costs, led to the lowest EY and EE in all perennial grasses and harvest times. These findings are in agreement with several studies dealing with the energy balance of perennial species in the Mediterranean area (Angelini et al., 2005; Mantineo et al., 2009; Amaducci et al., 2017).

At establishment, energy costs were influenced by irrigation (55.2%), fertilization (18.8%), transplanting (12.5%), harvesting (6.4%), soil tillage (5.6%) and weed control (1.5%). From the second year on, only harvesting costs affected crop management.

The biomass heating value of present perennial grasses (across the average of cultivation years) were similar to other perennial grasses grown in the same area in low-input conditions (Scordia et al., 2016).

A general increase from the second year was observed for both EY and EE, peaking at the third year, followed either by a decline (winter season) or a fairly constant trend (autumn season) at the fourth year.

In both winter and autumn regimes, the EY peak achieved in *Saccharum* (452 and 407 GJ ha^{-1}) is well comparable to *M.* x *giganteus* grown in the south Mediterranean under high-input conditions; it was higher than *Cynara cardunculus* at any condition of input supply, but lower than *A. donax* under high-input management (Mantineo et al., 2009). However, when *A. donax* was managed in low-input in the north Mediterranean, *Saccharum* EY was in line (Angelini et al., 2005).

On the other hand, the outstanding EE of *Saccharum* in both winter and autumn seasons (154 and 139 GJ ha^{-1}, respectively) was much higher than the EE of *A. donax* reported by Angelini et al. (2005), and comparable to *A. donax* and *M.* x *giganteus* values reported by Mantineo et al. (2009).

In the less productive post-establishment year (second year), *Saccharum* EY remained well above 200 GJ ha^{-1} yr^{-1}, higher than the findings of Monti et al. (2009) for *Panicum virgatum* fertilized with 200 kg N ha^{-1} yr^{-1}. In this second season, EE was in line with *M.* x *giganteus*, and higher than unfertilized *A. donax* and *P. virgatum* grown in the North Mediterranean (Amaducci et al., 2017).

The remaining species were not comparable to the other herbaceous perennial species studied worldwide for biomass production, except *Sorghum* when peaked at the third year in winter season (210 and 72 GJ ha^{-1}, for EY and EE, respectively).

This four-year study offered useful information on the impacts of cultivation practices at both establishment and post-establishment phases. However, further work is needed to study the yield persistence over a longer time period to get a better estimate of the whole crop life span net energy yield and efficiency.

5. Conclusions

This multiannual field study compared four autochthonous and undomesticated Mediterranean perennial grasses as novel candidate lignocellulosic bioenergy crops for the semi-arid Mediterranean area in autumn and winter harvest regimes. *Saccharum spontaneum* showed the highest productivity, averaging 20 Mg DM ha^{-1} $year^{-1}$ (in both harvest regimes) over 4 years, almost three-fold that of the next best – *Sorghum halpense*.

The modelled soil moisture highlighted that rainfed crops were subjected to severe water stress through most of the summer season in this environment. Beside the importance of rainfall amount, its distribution was the main determinant of yields and related efficiencies.

Winter harvest represents a preferable management strategy, as overwinter sustains senescence, structural polysaccharide increase and ash decrease, improving biomass quality.

Native perennial grasses may represent suitable feedstock for both energy and non-energy application, upon which a Mediterranean region biorefinery could be built if significant environmental benefits can be proven through further work on ecosystem services, key phenotypic traits and optimization of cultivation practices (e.g. carbon sequestration, maintenance of biodiversity, soil erosion, non-invasiveness, propagation, flowering and senescence, yield persistence).

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