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Towards sustainable energy crop cultivation: Feasibility of biomethane production using a double cropping system with various sorghum phenotypes

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Abstract

In order to achieve a sustainable energy crops production, energy crops should not compete for land against feed and food crops production. One option towards sustainable energy crop cultivation is the use of double cropping systems with minimum tillage use and digestate as natural fertilizer, where in the same growing season a second crop for biomethane production is planted after a first crop used for feed/food. Different sorghum phenotypes have been evaluated in the present study as first and second crop in a double cropping system. The Principal Component Analysis of the various sorghum phenotypes showed that starch content positively affects the methane production. However, sorghum chemical composition did not influence the profitability of bioenergy production as much as the total solids biomass yields of the different sorghum phenotypes. The highest total solids biomass productive sorghum phenotype led to the highest methane hectare yield.

Keywords: Anaerobic digestion; Biochemical Methane Potential; Principal Component Analysis (PCA); Hectare methane yield; Double cropping; Sorghum.

1. INTRODUCTION

Intensive soil tillage and external inputs such as synthetic fertilizers and pesticides to maximize crop productivity has been often used in cereals monoculture. These agronomic practices lead to soil physical erosion, recalcitrance of agricultural chemicals on the land, the need for weed control, nutrient leaching, and loss of biodiversity with a resulting considerable use of fossil fuels.¹

Biomethane valorization from anaerobic digestion obtaining heat, electricity or transport biofuel contributes towards the targets for renewable energy production and greenhouse gas mitigation in many European countries. Within the available alternatives of organic feedstock suitable to be digested in biogas plants, energy crops show many advantages due to their energetic content.² Maize had represented for many years the most predominant energy crop for biogas production in Central Europe but its cultivation was often carried out increasing the arable land areas.^{2,3} Additionally maize utilization in biogas plants was roughly criticized in relation to the food versus fuel debate for its possible disturbance with food/feed production.⁴

The sustainable production of food and feed must be a priority and energy crops cultivation should not impair it. Double cropping consists in growing two crops in succession, in the same arable land and in the same growing season where the second crop is planted after the first crop is harvested.⁵ Double cropping has been proposed as a sustainable energy crops production system.⁶ Dale et al.⁷ proposed the *Biogasdoneright*[®] concept in which in a biogas oriented double cropping system, food/feed production as first crop was not only affected by the cultivation of the second crop for biogas production but promoted chemical fertilizer reduction. Considering that just about 65–80% of the carbon fed to the digesters is converted into biogas, a part of this residual carbon, in addition to other nutrients, is incorporated into the soil in stable

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form by digestate valorization as natural fertilizer. Combining the digestate utilization with reduced or no tillage practices the soil carbon levels can be increased with a global improvement of soil fertility.

Sorghum has been assessed and studied in the last years as energy crop for biomethane production.^{8,9} Sorghum is a C4 plant with high photosynthetic efficiency that exhibits a higher drought resistance due to the extensive root system that allows to an efficient use of water from deep soil layers and minor fertilization requirement in comparison to other crops for bioenergy production.^{1,10,11} The complexity of biomass structure is a major challenge during anaerobic digestion of lignocellulosic biomass for biomethane production. Wannasek et al.³ showed that the cultivation of sorghum is a promising option for biomethane production especially with regard to sustainable increase in land use efficiency. There are different varieties of sorghum available on the market but detailed information about their chemical composition, biomass yield and methane hectare yield is limited, and especially scarce when sorghum is cultivated as second crop as feedstock in anaerobic digestion.

The aim of this study is to evaluate different sorghum phenotypes for biomethane production when cultivated as first crop and second crop in a double cropping system. The main novelty of this paper is the evaluation of a double cropping system for biomethane production using Principal Component Analysis to correlate chemical composition measured by Near infrared reflectance spectroscopy (NIRS) and Biochemical methane potential (BMP) tests.

2. MATERIAL AND METHODS

2.1. Sorghum cultivation, harvest and sampling

The agronomical trials were carried out at two experimental field sites placed in Emilia Romagna region in Italy named STUARD Experimental Farm (San Pancrazio - Parma)

1 and BETA Scarl (Baura and Contrapò - Ferrara). The trials examined the sowing of
2 different varieties of sorghum as first and second crop in both experimental sites. The
3 choice of commercial sorghum varieties was done on the basis of their maturation cycle,
4 location of experimental field site and climate in order to be able to harvest the crops at
5 four different growth stages up to the physiological maturity of plants.¹²

6 The commercial sorghum varieties used in the present study were classified in
7 four group on the basis of their phenotype, as reported in Shoemaker and Bransby.¹⁰
8 Each phenotype was identified by abbreviation from S1 to S4: forage (S1), high-
9 tonnage energy (S2), sweet (S3) and grain (tall size) (S4). The field experiments were
10 carried out as a randomized block design with four replicates for each variety of
11 sorghum cultivated as first crop and second crop at four growth stages in the two
12 experimental sites, as shown in Table 1.

13 It is important to remark the crops previously used in the two experimental field
14 sites to give an exhaustive overview of the rotation cycles. The previous crops in
15 STUARD experimental farm were cabbage with sorghum as first crop and forage barley
16 with sorghum as second crop. The previous crops in BETA were sequentially triticale
17 and sorghum with sorghum as first crop and triticale with sorghum as second crop.

18 In STUARD Experimental Farm the sowing was on May 23rd and on June 13rd
19 when sorghum was cultivated as first crop and second crop respectively. In such
20 experimental site the distance between the single rows in each plot was 0.45 m in both
21 cases. In BETA the sowing was on May 16th and on May 22nd when sorghum was
22 cultivated as first crop and second crop respectively. In such experimental site the
23 distance between the single rows in each plot was 0.75 m in both cases. The depth of the
24 sowing was 0.02-0.03 m.

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1 All experimental plots showed a silty clay loam soil texture. Tillage by plough
2 and subsoiler and secondary tillage were used with sorghum cultivated as first crop
3 while minimum tillage by harrow and grubber were performed with sorghum cultivated
4 as second crop.

5 STUARD and BETA experimental field sites were characterized by an average
6 cumulative rainfall within the sorghum vegetation period of 208 and 214 mm,
7 respectively. The abundant rainfall and the optimal temperature monitored daily during
8 the experimental activity established a sufficient water supply and for this reason
9 irrigation during the growing of sorghum was not necessary.

10 In STUARD Experimental Farm the fertilization was carried out on growing
11 crops with 100 kg N/ha of urea when sorghum varieties were cultivated both as first
12 crop and second crop. In BETA the fertilization was carried out on growing crops with
13 90 kg N/ha of urea when sorghum varieties were cultivated both as first crop and second
14 crop. In BETA, sorghum as second crop was additionally fertilized in pre-sowing with
15 20 m³/ha of liquid fraction of digestate. In BETA, 6 liters ha⁻¹ of CICLON® (Glyphosate
16 360) was used as herbicide before the sowing of sorghum cultivated as first crop.

17 The harvesting of sorghum took place in four moment at different growth stages
18 depending on the variety. When sorghum was cultivated as first crop the first harvest
19 was carried out between 94 and 96 days after the sowing, the second harvest between
20 111 and 112 days after the sowing, the third harvest between 126 and 132 days after the
21 sowing while the fourth and latest harvest was done after 145 days after the sowing.
22 When sorghum was cultivated as second crop, the first harvest was carried out between
23 94 and 96 days after the sowing, the second harvest between 107 and 112 days after the
24 sowing, the third harvest between 116 and 126 days after the sowing while the fourth
25 and latest harvest was done after 136 days after the sowing.

The plants height from the soil surface to the base of the tallest leaf was measured on three plants per harvested area immediately before each harvest. At the moment of harvest the growth stage, the biomass yield, i.e. harvested wet weight per harvested area, were also determined and recorded. The identification and the description of sorghum growth stage were done adapting the general Zadocks Growth Stages (Zadocks GS) scale for cereals to sorghum.

Sorghum was harvested and crushed to an average particle size of 2.5 cm by a plot forage harvester and the net size of each harvested area varied from 7 to 15 m² depending on the experimental site (STUARD or BETA) and operational conditions. The harvested samples were dried within 24 hours at 65°C until constant weight and grounded at 0.5 mm texture using a mill to determine the total solids of biomass. The dried and milled sorghum samples were store in airtight zip-lock bags at room temperature until laboratory analysis. Within the overall experimental time, 64 conditions were collected and processed for chemical composition determination by near infrared reflectance spectroscopy (NIRS) while in 56 of them the specific methane yields and methane production rate were additionally measured by biochemical methane potential (BMP) tests (Table 1).

2.2. Near infrared reflectance spectroscopy (NIRS)

Quadruplicates of all 64 conditions were analyzed for chemical components characterization using the near infrared reflectance spectroscopy NIRS (Table 1). In particular total solids (TS), ash, crude protein (CP), starch, sugars, lipids, neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) were predicted. The predicted values of NDF, ADF, and ADL concentration were used to calculate hemicellulose (HCEL) by subtracting ADF from NDF and cellulose (CEL) by subtracting ADL from ADF.

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The samples enclosed in spinning ring cups (36 mm inside diameter) were scanned on Foss NIRSystem 5000 monochromator in the 1100-2498 nm spectral region. Spectra recorded as $\log 1/R$, where R is the reflectance, were taken at 8 nm intervals using 173 wavelength. Mathematical treatments of spectral data were performed with the WinISI II V1.5 software (Infrasoft International, Port Matilda, PA, USA), using first order derivative with gaps and smoothing each 4 data point and standard normal variate and detrend procedure (SNVD) was applied for scatter correction. The Modified Partial Least Squares (MPLS) regression technique was used to develop the NIRS calibrations. The statistics used for the accuracy assessment of calibration equation were the standard error of calibration (SEC) and the coefficient of determination for the calibration (R^2); the statistic used for the accuracy assessment of prediction equation were the standard error of cross-validation (SECV) and the coefficient of determination for cross validation ($1-V_r$).

2.3. Biochemical methane potential (BMP) test

A total of 31 sorghum phenotype cultivated as first crop and 25 sorghum phenotype cultivated as second crop were analyzed for the determination of methane yield and methane production rate in order to have 6-8 conditions for each sorghum phenotype and distributed among the four harvests (Table 1).

Methane yields and methane production rate were evaluated by BMP tests according to standard UNI EN ISO 11734:2004 and described in Langone et al.¹³ The inoculum used in BMP tests was collected from an agricultural biogas plant (Reggio Emilia, Italy) and incubated at 38°C for 7 days prior to use. Glass bottles with 1.35 L working volume with 0.5 ratio between volatile solids (VS) from substrate and volatile solids from inoculum were used. BMP tests were run at 38°C in a thermostatic room for about 27 days. Biogas production was quantified by manometric system measuring the

increase of headspace pressure increasing in the bottle headspace. The methane content of the produced biogas is determined by gas analyzer (Model Gasboard-3200 biogas analyzer, Wuhan Cubic Optoelectronics Co., Ltd) based on Non-Dispersive Infrared technology for methane (CH_4) and carbon dioxide (CO_2) gases and electrochemical sensors for O_2 and H_2S gases. Productivity of inoculum is calculate separately by inoculum-only bottle (blank). The net methane production was obtained from the difference between the methane measured in the bottle with sorghum as substrate and the blank one. Specific methane yield was calculated by dividing the methane volume (L_N) by the quantity of the sample added to each bottle (kg_{VS} added) and it was expressed as normal litres of methane per kilogram of the volatile solids ($L_N \text{ kg}_{\text{VS}}^{-1}$). Specific methane production rate is calculated by dividing the methane yield by the corresponding time unit (day) and it is expressed as normal litres of methane per kilogram of the volatile solids produced in the time unit ($L_N \text{ kg}_{\text{VS}}^{-1} \text{ day}^{-1}$). The maximum value of specific methane production rate (R_{max}) reached during the BMP test was used to describe the kinetic of the biological process.

The methane hectare yield for each sorghum phenotype was calculated relating the specific methane yield measured by BMP tests with the biomass yield data obtained from field experiments and the chemical characteristics evaluated by NIRS analysis.

2.4. Principal Component Analysis (PCA)

Principal Component Analysis (PCA) was performed to investigate how chemical parameters could affect the methane production reducing the interrelated effect of the variables . Principal Component Analysis was applied to the dataset of 56 conditions where both NIR spectroscopy analysis and BMP tests were carried out to disclose the relations among the variables, particularly between chemical components, methane

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1 yields and methane production rate (SPSS Statistic software, SPSS Inc., Chicago,
2 USA).

3 **3. RESULTS AND DISCUSSION**

4 **3.1. Biomass yield**

5 *3.1.1. Sorghum cultivated as first crop*

6 When sorghum was used as first crop, the highest biomass yields were obtained with the
7 last harvest at 145 days after sowing (Fig. 1A). At that time S1 and S2 were in the early
8 dough growth stage (Zadocks GS 83) in which nutrients uptake from soil is almost
9 complete and lower leaves lose their functionality due to remobilization of nutrients to
10 the upper part of plant. In this early dough growth stage, S1 and S2 reached a biomass
11 yield of 99±16 and 97±25 Mg ha⁻¹, respectively (Fig. 1A). At the same time of harvest,
12 i.e. 145 days, S3 and S4 showed a biomass yield of 82±10 and 59±17 Mg ha⁻¹,
13 respectively (Fig. 1A), reaching the physiological maturity growth stage (Zadocks GS
14 89) in which grain should achieve its maximum dry weight.

15 The biomass total solids yields of harvested S1-S4 sorghum ranged from
16 10.9±1.8 to 29.0±9.0 Mg_{TS} ha⁻¹ within the different growth stages (Fig. 1B). The
17 highest biomass total solids yield was obtained during the last harvest of S2, which was
18 in its early dough growth stage while the lowest biomass total solids yield was obtained
19 during the first harvest of S4 in its early milk growth stage (Zadocks GS 73) (Fig. 1B).
20 The highest biomass total solids yield obtained with S2 was 16%, 33%, 75% higher
21 than those obtained with the cultivation of S1, S3, S4 respectively (Fig. 1B).

22 Low temperatures and drought during early growth stages could induce several
23 physiological dysfunctions in sorghum carrying in many cases to limited biomass yield
24 in terms of both wet weight and total solids.¹⁴ In this study, the rainy summer period
25 and the mild temperature in both field sites played an important role to facilitate the

vegetative growth of all S1-S4 sorghum as first crop reaching biomass yields comparable with other studies.^{2,3}

When sorghum was cultivated as first crop, the shortest height was observed in S4 in the interval from 1.94 to 2.03 m without significantly increasing from early milk to physiological maturity growth stages (Zadocks GS 73 to 89, respectively) (Fig. 1C). The tallest height was reached in S2 in the anthesis, early milk, early dough maturity growth stage (Zadocks GS 61, 73, 83, respectively) when sorghum plants grown up over 4.5 m (Fig. 1C). The big size of S2 permits to produce a huge amount of biomass yield with reduced grain quantity and this characteristic is typically observed in several high-tonnage sorghum phenotypes. High-tonnage sorghum (S2) is taller than grain sorghum (S4) that normally shows short panicles and panicle branches (Harlan and de Wet, 2010). S2 was leafier and late maturing in comparison to S4 as confirmed in other studies where forage sorghum (S1) and grain sorghum (S4) were investigated.¹⁵ The big size of high-tonnage sorghum (S2) may lead to an increase in stem lodging that could bring to serious problem during the mechanical harvesting of the crop. For this reason the good lodging resistance is one of the major agronomic trait necessary for biomethane sorghum production.¹⁶ The recorded heights of S1 and S3 were in the interval between 2.83 and 3.59 m that means a very similar and comparable size in correspondence with the same time of harvest (Fig. 1C). These morphological traits reflected the characteristics of the cultivated sorghum phenotype according with other studies where forage sorghum was cultivated reaching interval between 1.5-3.0 m.¹⁷ S1 and S3 showed an average height in the middle between S2 and S4 (Fig. 1C).

The optimal total solids content to ensile cereals with the purpose of biomethane production should be in the interval between 27-35%³ to have good quality silage and a

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1 stable silo with a limited loss of energy. In general, sorghum should be harvested as
2 soon as the lower leaves of the plant show signs of drying out.

3 When S2, S3, S4 were cultivated as first crop the total solid content of the
4 harvested plants ranging between 27-30% from early milk to physiological maturation
5 growth stages, while the optimal total solids content for ensiling was not reached in the
6 previous growth stages (Fig. 1D). The 27% in total solids content lower limit was not
7 achieved in S1 during the last harvest in early dough growth stage neither in STUARD
8 nor in BETA. A total solid content below 27% often could allow the loss of organic
9 substances (up to 10% in wet weight) due to a significant production of liquid silage
10 effluent¹⁸ and for this reason sorghum should be harvested with the cited minimal total
11 solids level when used for biomethane production.

12 3.1.2. *Sorghum cultivated as second crop*

13 When sorghum was cultivated as second crop, the highest biomass yields were obtained
14 during the 3rd harvest at 126 days after sowing with S2 reaching a biomass yield of
15 60±11 Mg ha⁻¹ in the anthesis growth stage (Fig. 1E). The highest S2 as second crop
16 biomass yield was about 38% lower in comparison to that obtained when S2 was
17 cultivated as first crop. Independently from the growth stage, the biomass yields of S1,
18 S2, S3, S4 as second crop were on average 48%, 31%, 44%, 29% lower than first crop,
19 respectively (Fig. 1E).

20 The biomass total solids yields of harvested S1-S4 as second crop ranged from
21 8.0 to 16.0 Mg_{TS} ha⁻¹ within the different growth stages. The highest biomass total
22 solids yield was obtained with S2 in early milk growth stage while the lowest biomass
23 total solids yield was obtained with the S4 in anthesis growth stage. The highest
24 biomass total solids yield obtained with S2 was 15%, 3%, 38% greater than those
25 obtained with the cultivation of S1, S3, S4 respectively (Fig. 1F).

When sorghum was cultivated as second crop, the shortest height was observed in S4 in the interval from 1.72 to 1.83 m (Fig. 1G). S4 cultivated as second crop showed a similar height observed in S4 as first crop identifying the size of PR845F sorghum as stable morphological trait when this variety was cultivated in double cropping system. The tallest height was reached in S2 in the anthesis growth stage when plants grown up to 3.98 ± 0.25 m; the average height of S2 as second crop was about 14% lower than the height of S2 as first crop. The recorded heights of S1 and S3 were in the interval between 2.79 and 3.77 m that means a very similar and comparable size with S2 as second crop and S3 as first crop (Fig. 1G).

When sorghum was cultivated as second crop the optimal total solids content for ensiling (27-35%) was reached at different growth stages in all the studied phenotypes identifying in Sugar Graze II, Hannibal, Freya and PR845F suitable sorghum varieties for second crop cultivation (Fig. 1H). In general the total solids content of S1-S4 at the time of harvest showed increasing values among the growth stages that lead to have the higher total solids biomass yield between early dough and physiological maturity with sorghum cultivated both as first crop and second crop.

3.2. Chemical composition

The chemical composition of S1-S4 averaged among growth stages is shown in Fig. 2A and Fig. 2B when sorghum was cultivated as first crop and second crop, respectively.

Ash content in S1-S4 phenotypes of 7.2-8.7 %TS observed in the first crop was not significantly different to those in the second crop in the interval 6.9-8.6 %TS. Similar ash concentration of 6.1-8.4 %TS in sorghum plants for biomethaneproduction is reported in Mahmood et al.² The slightly variations observed within S1-S4 phenotypes could depend on environmental factors such as a diverse mobilization of minerals to the soil before harvest as well as genetic and physiological differences

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among phenotypes.¹⁹ Average ash concentration reached higher values in S4 when cultivated both as first crop (8.7 ± 0.6 %TS) and second crop (8.6 ± 0.6 %TS) when compared with the other sorghum phenotypes. Particularly, PR845F variety exhibited the same ash concentration showing how the cropping system did not notably affect this chemical parameter. Anaerobic digestion is negatively impacted by ash content because it is not converted in biogas by microorganisms. The ash content should be as lower as possible reducing its introduction also by a good growing and harvesting process besides natural presence.²⁰

The CP content ranged between 5.2-6.5 %TS and 5.4-7.0 %TS when S1-S4 were cultivated as first crop and second crop, respectively. CP measured in this study was comparable with others reported in the literature.^{21,22} CP decreased with the advancing development of sorghum among growth stages up to early dough and physiological maturity.²³ CP reached higher value in S4 when cultivated both as first crop (6.5 ± 1.4 %TS) and second crop (7.0 ± 1.4 %TS) in comparison to S1-S3. The remarkable CP content of 6.5 ± 1.5 %TS with S1 as second crop could be explained with the fact that the leaves in sorghum contain the highest percentages of CP and the leaves development is a characteristic morphological trait in forage sorghum.

Lipids concentration ranged between 1.4-2.0 %TS in S1-S4 according to other studies reporting 0.3-2.5 %TS intervals.^{21,24} The lowest lipids content was 1.4 ± 0.3 %TS in S1. No significant differences in lipids were observed when sorghum was cultivated as first crop and second crop.

When sorghum was cultivated as first crop, the sugars ranged 10.0-14.6 %TS within intervals reported in Herrmann et al.²¹ S1 and S3 showed the highest average sugars content of 14.6 ± 2.7 %TS and 14.3 ± 2.9 %TS according with their forage and sweet phenotypes, respectively. The S1, S2 and S4 second crops followed similar trends

of sorghum as first crop with comparable sugars concentration. Surprisingly, S3 sweet sorghum exhibited the lowest sugars concentration when used as second crop. It is not clear why sugars content in S3 as second crop reached such low values, a possible explanation may be attributed to the diverse genetic background of Freya variety in comparison to Super Sile 20 variety. There is a natural variation for sugars concentration in stems within sorghum phenotypes for which grain sorghum accumulates little sugar in contrast to sweet sorghums that accumulate sugars up to 19% of total stem fresh weight.²⁵ The metabolic regulatory network plays a crucial role to drive carbohydrates production and accumulation in sweet sorghum and the study of Calviño and Messing²⁶ confirmed that it is more complex than previously expected. Despite the notorious differences in sugars content between grain and forage sorghum, genetic diversity studies did not support a clear clustering of sweet sorghum varieties and Freya seems to share the sugars trait with grain sorghum phenotype.

There were substantial differences in the starch content of S1-S4 according to their phenotype. The grain sorghum (S4) tended to have the highest starch concentration of 14.9 ± 6.8 %TS and 15.2 ± 8.7 %TS within first and second cropping system, respectively because carbohydrates are accumulated in the grain. The lowest starch concentrations of 3.6 ± 2.6 %TS and 3.9 ± 1.9 %TS were observed for S2 when cultivated both as first crop and second crop according to the fact that S2 is a high-tonnage sorghum which produce little or no grain. In general, starch increased with the advancing development of all S1-S4 sorghum among growth stages up to early dough and physiological maturity.²⁷

Starch and sugars are non-structural carbohydrates while cellulose (CEL) and hemicellulose (HCEL) represent mainly sorghum structural carbohydrates. CEL and HCEL varied significantly among S1-S4 phenotypes. When cultivated as first crop, S2

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showed on average the highest amounts of CEL and HCEL of 35.9 ± 2.3 %TS and 21.6 ± 1.6 %TS, respectively. Analogously, S2 exhibited similar values and trends if used as second crop (CEL= 35.8 ± 2.5 %TS; HCEL= 21.4 ± 1.4 %TS). In both cropping system, the lowest CEL and HCEL concentration were observed in S4 with the same concentration of 27 %TS and 19 %TS, respectively confirming that PR459F was not affected by diverse cropping system. The sum of structural carbohydrates content was on average $S2>S1>S3>S4$ for the first cropping while the $S2>S3>S1>S4$ pattern was detected for the second cropping. Super Sile 20 and Freya varieties were clustered into sweet sorghum phenotype but the question of how and when did the CEL and HCEL trait appeared within certain varieties of sweet sorghum remains open question linked to the carbohydrate metabolizing enzymes during stem development (Calviño and Messing, 2012). Even though Super Sile 20 and Freya showed several typical common morphological traits within sweet sorghum phenotype, they seem be also closer to grain sorghum (S4) and high-tonnage sorghum (S2), respectively exhibiting intermediate structural carbohydrates concentration of such phenotypes.

Acid insoluble lignin (ADL) ranged 3.2-5.2 %TS and 3.3-4.9 %TS when S1-S4 were cultivated as first crop and second crop, respectively. The ADL concentration was similar to those reported in other works in the interval of 3.5-11.2 %TS.^{11,21} The lowest ADL concentration was observed in BMR 333 variety probably because the brown mid-rib (BMR) sorghum genetic background contain lower lignin content compared with the other non-BMR varieties.

3.3. Specific methane yields

Specific methane yields of S1-S4 ranged from 244 up to 279 $L_N \text{ kg}_{VS}^{-1}$ and from 268 up to 295 $L_N \text{ kg}_{VS}^{-1}$ among the growth stages when sorghum was cultivated as first crop and second crop, respectively.

The highest average specific methane yields were measured for S4 in both cropping systems while lowest average methane yields were obtained for S1 as first crop and for S1 and S3 when sorghum was cultivated as second crop (Fig. 3). In general, none reduction in specific methane yields was observed with sorghum as second crop, on the contrary an improvement of about 10% was measured in S1 and S2.

The specific methane yields obtained in this work were a little bit lower than other data reported in literature. Herrmann et al.²¹ reported the utilization of sorghum as a potential substitute for maize for biomethane production when cultivated as first or second crop reaching 248–348 L_N kg_{VS}⁻¹. In Wannasek et al.³ methane yields of three vegetation periods of five sorghum varieties within several growth stages between 20 and 45% TS were reported to be in the interval 290–420 L_N kg_{VS}⁻¹. Mahmood et al.² found the methane yields of 14 sorghum varieties ranged between 250 and 354 L_N kg_{VS}⁻¹. Often sorghum samples investigated in literature were analyzed as fresh or ensiled samples while in this study the BMP tests were carried out with dried and milled samples for the comparative evaluation of several sorghum phenotypes limiting the interferences of particles size in the test. Drying and milling could lead to losses or alteration of organic compounds justifying the lower methane yields besides genotype and cultivation conditions observed in comparison to the cited works. Such kind of pretreatment of sorghum samples could have a significant impact on methane yield restraining the comparability of this study with other works as suggested in Raposo et al.²⁸

3.4. Methane production rate

Maximum specific methane production rate ($R_{\max}$) of S1–S4 ranged from 29.7 up to 38.5 L_N kg_{VS}⁻¹ day⁻¹ and from 34.8 up to 41.1 L_N kg_{VS}⁻¹ day⁻¹ when sorghum was cultivated as first crop and second crop, respectively. Highest $R_{\max}$ was measured with

S4 in early dough growth stage and physiological maturity in first cropping and second cropping, respectively. (Fig. 4).

The lowest average R_{max} were obtained with S1 and S2 when sorghum was cultivated as first crop (Fig. 4). In general, none reduction in R_{max} was observed with sorghum as second crop. Not significant differences on R_{max} between first and second crop, as observed in the preset study, was reported in Herrmann et al.²¹ where the R_{max} for forage sorghum ranged 53–175 and 66–125 $L_N\ kg_{VS}^{-1}day^{-1}$ when cultivated as first crop and second crop, respectively.

3.5. Effects of chemical composition on methane yields and methane production rate

The dataset of 56 conditions analyzed by NIRS for chemical parameters determination and by BMP tests for their specific methane production and R_{max} was processed through PCA. The first two principal components explained about 75.36 % of the total variance. The first principal component PC1 alone explained 42.06 % of the total variance, mainly due to variation in CEL, HCEL, ADL and starch but in the opposite direction respect to structural carbohydrates and ADL (Fig. 5). The PC2 accounted for 33.29% of the total variance and it was dominated by chemical components such as lipids and sugars but in the opposite direction. Also specific methane yield and R_{max} contributed to explain the variance in PC2.

These results indicate that starch, lipids and sugars are the most relevant parameters that affect methane yield and methane production rate in sorghum. Regarding the examined chemical parameters, only CP variance was not explained by the extracted components and for this reason this was not considered a suitable parameter for PCA. This finding is in accordance with the work of Herrmann et al.²¹ where CP of several energy crops was considered a chemical parameter with a weak

correlation for methane yields and $R_{\max}$ but with positive effects. In other cases the CP content of 15 varieties maize silage used as energy crop was argued as an important chemical parameters in anaerobic digestion for methane production.²⁹

In this study, starch and lipids revealed the closest positive correlation between chemical composition with methane yield and methane production rate. Lipids and starch are more easily converted into methane than structural carbohydrates CEL and HCEL facilitating acidogenesis, acetogenesis and methanogenesis rather than hydrolysis. Among variables, sugars and lipids were highly independent and inversely correlated (Fig. 5).

The enzymatic hydrolysis of CEL is mainly influenced by its crystallinity, degree of polymerization, and its accessible surface area. HCEL is often a connection between ADL and CEL fibers and give rigidity to the whole lignocellulosic matrix. Furthermore, ADL is cross-linked with other cell wall components decreasing the anaerobic degradability of such kind of feedstock. Herrmann et al.²¹ confirmed in their investigation the significant negative correlations between CEL and HEM and specific methane yields of several energy crops.

In general the CEL and HCEL are mainly degraded during the first phases of anaerobic digestion, i.e. hydrolysis and acidogenesis, while the ADL fraction is very challenging to be broken down. In this work ADL affected weakly the specific methane yields and $R_{\max}$ as expected for this kind of chemical parameters. The ADL fraction is very difficult to degrade for anaerobic digesters microbial population and, typically, a relative accumulation of lignin is observed during anaerobic digestion due to degradation of other chemical components such as CEL and HCEL.³⁰ Probably such kind of lignocellulosic matrix impacts negatively the hydrolysis kinetic or any microbiological stages preceding the methanogenesis and consequently the $R_{\max}$

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measured in this study. The R_{max} of S1 and S2 cultivated as first crop exhibited lower values than S4 supporting the hypothesis that the degradation of the lignocellulosic matrix could be a rate limiting step in the microbiological process.

The distribution and composition of ADL are extremely important for microbial enzyme accessibility and the digestibility of feedstock for biomethane production. Sometimes pretreatment technology are necessary at full-scale to improve the co-digestion of lignocellulosic feedstock by hydrolytic microorganisms to obtain a better methane production efficiency in the digesters. It can be done by increasing the specific surface area that is exposed to microorganism action by physical, chemical, or biological methods.³¹

3.6. Methane hectare yield

The methane hectare yield of land cultivated with a certain energy crop is one of the main parameters affecting the use of that crop as substrate for anaerobic digestion .^{3,32,33} For farmer interested in biomethane production, the final goal is to reach the maximum methane hectare yield possible.³⁴

Figure 6 illustrates the methane hectare yield in terms of methane production per unit of cultivated land area when S1-S4 reached the optimal total solids for the storage of feedstock as silage at time to harvest. It was calculated a methane hectare yield from 3879 up to 7492 m³CH₄ ha⁻¹ and from 3137 up to 4630 m³CH₄ ha⁻¹ when sorghum was grown as first crop and second crop, respectively (Fig. 6). In both cases, S2 high-tonnage sorghum exhibited the highest methane hectare yield in comparison to other sorghum phenotypes (Fig. 6). Although S4 exhibited higher specific methane production and R_{max} than S2 caused by greater S4 content in starch, lower amount of structural carbohydrates (CEL, HCEL) and limited ADL level compared to S2 (Fig. 2, Fig. 3, Fig. 4), the calculation of methane hectare yield lead to address S2 as the best

sorghum phenotype for biomethane production instead of S4 (Fig. 6). The biomass total solids yield obtained with S2 as first crop was 16%, 33%, 75% higher than those obtained with the cultivation of S1, S3, S4 respectively. Similarly in the second cropping the S2 highest biomass total solids yield was 15%, 3%, 38% higher than those obtained with the cultivation of S1, S3, S4 respectively.

Additionally to the highest methane hectare yield observed for S2 in both first and second crops, S3 sweet sorghum as first crop exhibited the second highest methane hectare yield reaching $5689 \text{ m}^3\text{CH}_4 \text{ ha}^{-1}$. These results highlight how S2 and S3 sorghum phenotypes could be suitable substrates in biogas plants when compared with a reference crop maize methane hectare yield of about $6000 \text{ m}^3\text{CH}_4 \text{ ha}^{-1}$.^{3,35}

Biomass total solids yield and not chemical composition is a much more crucial parameter for choosing a sorghum phenotype for biomethane production. Nevertheless, other parameters not evaluated in this study might influence the selection of a certain sorghum phenotype for biomethane production, e.g. big size of a certain sorghum may lead to an increase in stem lodging that could bring to serious problem during the mechanical harvesting of the crop. A good lodging resistance is a major agronomic trait necessary for biomethane sorghum production.

4. CONCLUSIONS

Biomethane production using double cropping system with sorghum is a promising option for sustainable energy crops cultivation. Starch and lipids revealed the closest positive correlation between composition with methane yield and production rate. However, biomass total solids yield and not composition is a much more crucial parameter for choosing a sorghum phenotype for biomethane. According to EU Renewable Energy Directives (RED II), biomethane production from sorghum can be

considered an advanced biofuel when cultivated in double cropping system avoiding land competition versus food/feed markets and it can be counted to reach the targets for renewable energy sources consumption to 2030.

E-supplementary data of this work can be found in online version of the paper

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Figure captions.

Figure 1. Biomass yield, biomass total solids yield, height and total solids at time of harvest of S1-S4 Sorghum phenotypes as a function of growth stages. Data are showed as average of four replicates in each experimental field sites (STUARD and BETA). Zadock Growth Stage: 49= booting; 55= inflorescence emergence; 61= anthesis; 73=early milk; 77= late milk; 83=Early dough; 87=Hard dough; 89=physiological maturity.

Figure 2. Chemical composition of S1-S4 phenotypes averaged of the different growth stages when Sorghum was cultivated as first crop (A) and second crop (B). Values are expressed as percentage of Total Solids (%TS). CP – crude protein; CEL – cellulose; HCEL – hemicellulose; ADL – acid detergent lignin.

Figure 3. Comparison of specific methane yields of S1-S4 Sorghum phenotypes. Data are shown as average and standard deviation of the different growth stages when Sorghum was cultivated as first crop (dark gray) and second crop (light gray).

Figure 4. Comparison of maximum production rate (R_{max}) of S1-S4 Sorghum phenotypes. Data are shown as average and standard deviation of the different growth stages when Sorghum was cultivated as first crop (dark gray) and second crop (light gray).

Figure 5. Loadings plot for the first principal component (PC1) versus the second principal component (PC2). CP – crude protein; CEL – cellulose; HCEL – hemicellulose; ADL – acid detergent lignin; CH_4 yield – specific methane yield; R_{max} – maximum methane production rate.

Figure 6. Comparison of methane hectare yields S1-S4 sorghum phenotypes harvested at optimal total solids content for ensiling (27-35 %TS). Total solids content of Sorghum at time to harvest is shown on the bottom of bars.

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Table 1. Commercial sorghum varieties used in the two experimental field sites (BETA and STUARD) and harvest time. Grey colors indicate conditions that has been tested for biomethane production.

	Phenotype	Genetic background*	Variety	Supplying company	Cropping	Experimental field site	Harvest			
							1 st	2 nd	3 rd	4 th
S1	Forage	<i>S. bicolor</i> X <i>S. sudanense</i> (BMR)	BMR 333	Società Italiana Sementi (SIS)	First	BETA				
						STUARD				
		<i>S. bicolor</i> X <i>S. sudanese</i> X <i>S. saccharatum</i>	Sugar Graze II	Padana Sementi	Second	BETA				
						STUARD				
S2	High-tonnage	<i>S. bicolor</i> X <i>S. bicolor</i>	Bulldozer	KWS	First	BETA				
						STUARD				
		<i>S. bicolor</i> X <i>S. bicolor</i>	Hannibal	KWS	Second	BETA				
						STUARD				
S3	Sweet	<i>S. bicolor</i> X <i>S. saccharatum</i>	Super Sile 20	Caussade Semences	First	BETA				
						STUARD				
		<i>S. bicolor</i> X <i>S. sudanense</i>	Freya	KWS	Second	BETA				
						STUARD				
S4	Grain (tall size)	<i>S. bicolor</i> X <i>S. bicolor</i>	PR845F	Pioneer	First	BETA				
						STUARD				
		<i>S. bicolor</i> X <i>S. bicolor</i>	PR845F	Pioneer	Second	BETA				
						STUARD				

* Manufacturer specification

Figure 1. Biomass yield, biomass total solids yield, height and total solids at time of harvest of S1-S4 Sorghum phenotypes as a function of growth stages. Data are shown as average of four replicates in each experimental field sites (STUARD and BETA). Zadock Growth Stage: 49= booting; 55= inflorescence emergence; 61= anthesis; 73=early milk; 77= late milk; 83=Early dough; 87=Hard dough; 89=physiological maturity.

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Figure 2. Chemical composition of S1-S4 phenotypes averaged of the different growth stages when Sorghum was cultivated as first crop (A) and second crop (B). Values are expressed as percentage of Total Solids (%TS). CP – crude protein; CEL – cellulose; HCEL – hemicellulose; ADL – acid detergent lignin.

190x70mm (150 x 150 DPI)

Figure 3. Comparison of specific methane yields of S1-S4 Sorghum phenotypes. Data are shown as average and standard deviation of the different growth stages when Sorghum was cultivated as first crop (dark gray) and second crop (light gray).

89x54mm (150 x 150 DPI)

Figure 4. Comparison of maximum production rate (R_{max}) of S1-S4 Sorghum phenotypes. Data are shown as average and standard deviation of the different growth stages when Sorghum was cultivated as first crop (dark gray) and second crop (light gray).

89x54mm (150 x 150 DPI)

Figure 5. Loadings plot for the first principal component (PC1) versus the second principal component (PC2). CP – crude protein; CEL – cellulose; HCEL – hemicellulose; ADL – acid detergent lignin; CH4 yield – specific methane yield; Rmax – maximum methane production rate.

149x86mm (220 x 220 DPI)

Figure 6. Comparison of methane hectare yields S1-S4 sorghum phenotypes harvested at optimal total solids content for ensiling (27-35 %TS). Total solids content of Sorghum at time to harvest is shown on the bottom of bars.

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