Utilizing Leafy genes as resources in quality silage maize breeding

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Abstract

The primary goal of silage maize production is to obtain the greatest possible amount of digestible nutrients. Although it is not of the highest value in terms of starch, leaves provide ruminants with sufficient amount of green mass and facilitate nutrient uptake. The nutrient and fibre content of leaves at harvest affect the quality of silage. In cooperation with Glenn Seed Ltd, the Agricultural Research Institute of HAS had an opportunity to use an inbred line carrying the dominant allele *Leafy1* (*Lfy1*). The number and surface area of leaves were measured in various hybrid combinations, and the hybrids and parents were compared morphologically. The combinations causing the highest heterosis were identified. Compared to lines without *Lfy1* gene and hybrids in the same maturity group, these lines had an additional 3-4 leaves above the ear on average, resulting in an extra 30-40% leaf area. The hybrid Siloking (Lfy) produced 50% more leaves above the ear than the standard, leading to an extra 40% leaf area. The results demonstrate that the increased total leaf area of Lfy silage hybrids and the number of leaves above the ear, ensure that new leaves with high sugar content capable of photosynthesizing can develop until the end of grain filling. The increased leaf-mass produced by Lfy hybrids may be utilized, bearing in mind the relevant guidelines by the European Union, also as biogas raw material in sustainable energy management.

Keywords: silage maize; *Leafy* gene; leaf area; biogas; *Zea mays* L

Introduction

According to the most recent data from FAO, grain and silage maize was grown globally on approximately 160 million hectares in 2010, producing a grain yield of 800 million tonnes. The biggest growers are USA and China, where around 60% of the global grain maize yield was produced (approximately 500 Mt). In the 27 member states of the European Union the grain maize area was eight million hectares and the total grain yield of 54–55 Mt.

The growing area data, however, does not give a picture of the ratio of silage maize grown on these areas. In major grain maize-producing countries this figure may be around 15% of the total growing area, while in the cooler, wetter countries of Western Europe it may be considerably higher. In France, the maize growing area is around three million hectares, on half of which 70 million tonnes of silage are produced to feed a cattle population of around four million. But mention could also be made of the Netherlands, Germany, Denmark or Poland, where a decisive proportion of the maize growing area is sown specifically for silage production. Unlike grain maize, the end-product of silage production must be utilised in the area where it is produced, as it is not feasible to export surplus quantities, and its use for other purposes has only become possible in recent years, since the green light was given for the production of "green energy" in a number of European countries. The fundamental aim of silage maize production is to produce the highest possible quantity of digestible fodder from unit area. When choosing the ideal type of silage hybrids, factors influencing the quality must be taken into consideration. As well as total dry matter production, the digestibility of the silage is also extremely important. In addition to the quantity of starch, the contents of sugar, protein, digestible fat, fibre and lignin are also decisive. Attention must also be paid to the grain/stalk ratio, the grain/leaf ratio, the stay-green character and the optimal time of harvest when the leaves are still green and the grains are not too ripened.

For many long years, efforts have been made all over the world to improve the quality of silage by breeding. The use of mutant genes, for example the *BMR*, *wx* and *floury-2* genes and the development of silage hybrids with high oil or high grain content have not resulted in satisfactory improvements because their poorer yield average and other negative agronomic traits (greater susceptibility to stalk, leaf and seed infection, less stable quality parameters, etc.) have inhibited their wider use. Intensive cattle farms geared to producing high quality meat and high milk yields require silage hybrids providing large fresh mass, to ensure maximum dry matter production per hectare with excellent digestibility.

Many authors reported a positive correlation between higher leaf number and an increase in bio-

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mass. Chase and Nanda (1967) found that the leaf area above the ear has a decisive role in the yield per hectare. This confirmed the results achieved by Kisselbach and Lyness (1945), Schmidt and Lovely (1959), and Schmidt and Colville (1967). Similar conclusions were also drawn by Berzy and Fehér (1995), Dwyer et al (1998), Andrews et al (2000), Subedi and Ma (2005), and Hammer et al (2009).

The mutant gene *Leafy1* (*Lfy1*), was discovered several decades ago by Donald L Shaver and Robert C Muirhead. The main advantage of this gene is that it increases the total leaf number, and particularly the number of leaves above the ear. Shaver (1983) was the first to report the results obtained with maize plants carrying this gene: extra leaves above the ear, lower ear attachment height and greater yield potential. The greater leaf area provided by the higher leaf number of leafy types, especially above the ear, results in a shorter vegetative period and a longer grain-filling period compared to the normal analogues (Begna et al, 1997; Modarres et al, 1997a), possibly the increased leaf area can provide assimilates for a longer time. In connection with the number of leaves above the ear, Begna et al (2001) reported that these genotypes have an average of two more leaves than their conventional counterparts. The effect of this substantial increase in active photosynthesising surface on the yield and on seed quality was discussed by Dodd (1977), Tollenaar and Dwyer (1990), Stewart and Dwyer (1993), Modarres et al (1997b), Begna et al (1999), and Dijak et al (1999). According to Hammer et al (2009) the change in canopy architecture had little effect, but probably had an important indirect effect via leaf area retention and the partitioning of carbohydrates to the ear. These authors consider that the size of photosynthesising area available during flowering and grain filling is the most decisive factor influencing the extent of biomass accumulation. The light-fixing ability of the canopy structure is most efficient in the case of high leaf area index (LAI). However, care must be taken to choose the optimal plant density to avoid shading. In this regard genotypes with erect leaves are better for high plant density. The effect of the *Lfy* gene on plant quality was analysed by Dronovalli (1992). There is a lower rate of parenchymal cell death in the internodes, resulting in stronger stalks, while the main leaf veins have a smaller diameter, which could have a positive effect on digestibility. In order to make the photosynthesising surface even more efficient, Modarres et al (1997a) crossed Lfy and dwarf (*rd1*) materials. The crossing combinations had greater leaf area and a faster growth rate. In theory this should make it possible for maize to spread to areas with a short vegetation period and to produce higher yields. Despite the higher leaf and root area of Lfy hybrids, Costa et al (2002) found in mineral fertilisation experiments that it was not necessary to apply higher N rates.

The yields of conventional silage maize genotypes were successfully increased by breeding, but little progress was made in improving quality, particularly digestibility (Lauer et al, 2001). This problem could be

Table 1 - Some morphological traits of 2 Lfy and 2 conventional hybrids (Martonvásár, 2002-2005, 2007)

Morphological traits	Limasil (Lfy)	Mv NK 333 (conventional)	Kámasil (Lfy)	Maxima (conventional)
	FAO 300		FAO 500	
Ear placement (cm) $LSD_{5\%}=3.21$	73.87	85.97	79.38	106.45
Plant height (cm) $LSD_{5\%}=3.12$	231.63	210.82	230.68	243.82
Leaf area above the ear (cm^2) $LSD_{5\%}=151.24$	3521.65	2644.28	3717.20	2319.28
Leaf area below the ear (cm^2) $LSD_{5\%}=174.96$	2370.08	2494.87	3240.92	4031.08
Total leaf area (m^2/ha)	24651.47	18509.94	26020.48	16235.10
Total leaf area (cm^2/plant)	5894.73	5139.15	6958.12	6350.36
Leaf number above the ear $LSD_{5\%}=0,30$	8.92	6.02	9.65	6.10
Leaf number below the ear $LSD_{5\%}=0,28$	5.18	5.98	6.10	7.68
Total leaf number	14.10	12.00	15.75	13.78

Figure 1 - Leaf area of the FAO 300 hybrids (LIMASIL - Lfy and MvNK 333 - conventional) and in the group FAO 500 (KAMASIL - Lfy and MAXIMA - conventional) above the ears

solved by cultivating Lfy hybrids (Dwyer et al, 1998; Sanavy at al, 2009; Hegyi, 2011). The quality benefits of increasing the leaf number and leaf area, i.e. the assimilation surface, were discussed by Dwyer et al (1995) and Stewart et al (1997). These authors compared the above-ear canopy structure of Lfy and non-Lfy hybrids, and also examined the distribution of soluble carbohydrate production and storage of insoluble carbohydrates in various leaf storeys. The mathematical model they elaborated could be a useful tool in Lfy breeding. In Lfy plants the soluble carbohydrate content in the above-ear parts was twice as high on average as in normal plants. A similar result was reported by Andrews et al (2000).

In connection with the digestibility of various plant organs, a high grain ratio was reported to be desirable in silage maize, first by Morrison (1956) and later by Adalena and Milbourn (1972). Perry and Caldwell (1969), on the other hand, found that assimilates not accumulated in the grain but stored in the vegetative parts were just as easily digestible as the grain. Experiments performed by Pinter (1986) and Pinter et al (1988) proved that, at a harvest index of over 35%, the energy concentration of the whole plant depended not on the grain ratio but on the quality traits of the vegetative organs.

Data from the literature also confirm that the area and mass of the leaves above the ear, which are the most valuable from the point of view of silage production, were much greater in Lfy than in non-Lfy hybrids, and this was combined with better quality. It is still in question whether this benefit can be utilized in every genetic background or environment.

In the present work the effect of the Lfy gene on the canopy structure was analysed for a period of nearly 10 years in experiments on a number of registered, commercially grown Lfy and non-Lfy Martonvásár hybrids and on their parental components in order to determine whether the large leaf area of the Lfy hybrids was really associated with any change in quality. A further aim was to assess the value of this type of hybrids for the production of renewable “green energy” (biogas).

Materials and Methods

Experiments were set up to compare the leaf areas of two leafy (LIMASIL, KAMASIL) and two non-leafy (MvNK 333, MAXIMA) maize hybrids from Martonvásár. The experiment was designed to compare different types of non-isogenic hybrids (Lfy and non-Lfy) within the same maturity group. The experiments were focused on the value of the different canopy structure above the ear of the Lfy and non-Lfy hybrids. The trials were set up between 2002 and 2010 in the nursery of the Agricultural Research Institute of the Hungarian Academy of Sciences in Martonvásár (47°18’ N, 18°46’ E) and in the “winter” nursery maintained by the institute’s Department of Maize Breeding in Buin, Chile (33°43’ S, 70°44’ W). The soil in Martonvásár was a chernozem, while the experiment in Chile was sown on an alluvial meadow soil. In both locations the experiments were laid out in a random block design with three or four replications, in plots with 20 plants per row, under irrigated conditions. The annual heat sum during the maize vegetation

Table 2 - Studies of the heterosis and heterobeltiosis on the 2 lfy hybrids and on their parental components (2 years' average, Martonvásár)

Genotype	Plant height (cm)	Leaf number below ear	Leaf number above ear	Leaf number total	Heterosis of leaf number above ear (%)	Heterobeltiosis of leaf number above ear (%)	Leaf area (cm^2/plant) below ear	Leaf area (cm^2/plant) above ear	Leaf area (cm^2/plant) total	Heterosis of leaf area above ear (%)	Heterobeltiosis of leaf area above ear (%)
P1 (Lfy)	154.60	5.00	8.32	13.32			1701.74	2615.00	4316.74		
P2	170.33	4.94	6.31	11.25			1652.83	1531.12	3183.95		
F1 (Kámasil)	229.75	6.03	9.94	15.97	35.89	19.47	2976.56	3683.23	6659.79	77.67	40.85
$LSD_{5\%}$	5.03	0.31	0.36	0.41			131.05	181.92	220.30		
P1 (Lfy)	154.60	5.00	8.32	13.32			1701.74	2615.00	4316.74		
P3	135.51	3.82	4.59	8.41			755.02	935.42	1690.44		
F1 (Limasil)	227.33	4.81	8.78	13.59	36.02	5.53	1797.89	3247.45	5045.34	82.93	24.19
$LSD_{5\%}$	4.55	0.34	0.45	0.56			120.32	176.74	209.96		

period was very similar at the two locations (1,300-1,400 heat units).

Based on a 30-year series of meteorological data of Hungarian (OMSZ) and Chilean (DMC) meteorological services, the average rainfall during the growing season (Apr-Oct) in Martonvásár is 310 mm, requiring supplementary irrigation, while in Chile (Nov-Mar) there is a much lower rainfall sum, amounting to only around 100 mm. At the same time, due to the greater height above sea level (+ 600 m) the difference in the day/night temperatures results in greater dew formation, though this is not sufficient to make rainfall supplies adequate. The extra water required for intensive maize production was provided in the form of ditch irrigation.

The experiments set up in Hungary and Chile between 2002 and 2010 were in agreement with data published in the literature. The two single cross Lfy hybrids were both developed in Martonvásár using the same Canadian line containing the Lfy gene as one of the parental components. The other components of these lines were Martonvásár inbred lines. The LIMASIL (Lfy) hybrid belongs to the FAO 300 maturity group and the KAMASIL (Lfy) hybrid to the FAO 500 group. These were compared with the non-Lfy silage hybrids Mv-NK 333 (FAO 300) and MAXIMA (FAO 500). The measurement of the leaf area was primarily performed manually, by removing the leaves individually and calculating the leaf area using the equation elaborated by Montgomery (1911). In Martonvásár, in addition to the manual method, the leaf area was also estimated using a portable leaf area meter (LI-3000A, LiCor, Switzerland) or a laboratory meter (Delta-T Devices, Cambridge, UK).

An experiment to study the biogas production of four Lfy (Limasil, Dunasil, Mv Siloking, Mv Massil) and four non-Lfy (Mv Maros, Mv NK 333, Mv TC 437, Maxima) hybrids was set up in four-replication trials in Martonvásár, Hungary in 2009 and 2010. The experiment was sown with a plant density of 80,000 plants per hectare. During August one row of each of the four Lfy and four non-Lfy varieties was cut, and chopped samples were prepared from the whole above-ground part of the plants. A part of each sample was used to analyse biogas yield in the BETA Research Institute in Sopronhorpács, Hungary. The other part of each sample was measured for chemical composition traits such as dry matter, lignin, starch, etc. The NDF (neutral detergent fibre) and WSC (water-soluble carbohydrates) contents were also recorded. The chemical composition of the silage

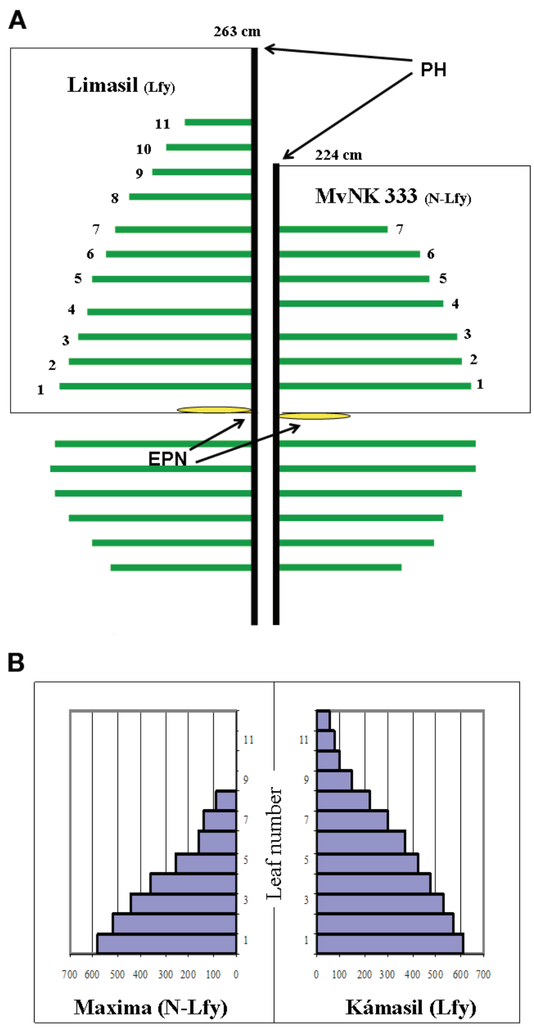

Figure 2 – Theoretical canopy structure: A) leaf length (cm) and B) leaf area (cm^2) of a Lfy and a non-Lfy (N-Lfy) hybrid (Martonvásár, 2002-2007). EPN: ear placement node; PH: plant height.

samples was analysed using a Fourier transform NIR spectrometer (Bruker, Ettlingen, Germany) and INGOT software. The quantity of biogas was measured in a 4-litre fermenter linked to a gas meter.

The biometrical analysis of the data was performed using the Agrobase'99 program.

Results and Discussion

Morphological comparison of Lfy and non-Lfy hybrids

Results of the morphological parameters recorded in the experiments are listed in Table 1. The leaf number per plant and the number of leaves above the ear were significantly larger for the Lfy hybrids than for the non-Lfy hybrids of the same maturity group for both the medium- and late-maturing hybrids. In the case of LIMASIL the increase in leaf number per plant averaged 3.9, resulting in a 33% greater leaf area above the ear compared with the conventional hybrid, while for KAMASIL there were 3.55 more leaves on average and a 60% greater theoretical leaf area above the ear than recorded for the normal hybrid in the same maturity group. With a plant density of 70,000 plants per hectare, this was equivalent to an assimilating leaf area of 2.6 hectares for KAMASIL and 2.4 hectares for LIMASIL on each hectare of land.

Several years of observations on these Lfy hybrids revealed that they have relatively poor tolerance of drought and heat stress, but under intensive, irrigated conditions they consistently produce this quantity of extra assimilating leaf area above the ear, resulting in enhanced leaf-mass production compared to normal hybrids (Figures 1, 2).

Comparison of Lfy hybrids and their parental components

The two Lfy hybrids KAMASIL (P1 x P2) and LIMASIL (P1 x P3) were compared with inbred line P1 (Lfy line : GL 62), used as female parent in both hybrids, and with the two non-Lfy Martonvásár inbred lines used as male parents (P2: HMv 5405; P3: HMv 2541). For both hybrids the leaf area above the ear was considerably greater than the average of the parental components. The extent of heterosis for P1 + P2 was + 77,67% for KAMASIL and P1 + P3 was + 82,93% for LIMASIL, while the extent of heterobeltiosis, compared with the better parent (P1), was + 40,85 and + 24,19%, respectively (Table 2).

Quality traits of Lfy and non-Lfy hybrids

The leaves above the ear form one of the most valuable parts of Lfy hybrid plants. These are more abundant and also have a significantly greater sunlight-absorbing surface than those of conventional hybrids. Prior to ensiling and before complete physiological maturity, there are still a large number of fresh leaves that contain greater quantities of sugar than those of normal hybrids. The results obtained in 2010 support this theory, especially those obtained for the Lfy hybrid KAMASIL (Table 3). The Lfy hybrids were also found to have a lower lignin content, which may also have a positive effect on fermentability and digestibility.

Lfy hybrids as a source of biogas

Biogas formation consists fundamentally of two processes, fermentation and methane formation. During the phases of fermentation (hydrolysis, acidic phase) the large-molecule organic matter is decomposed with the help of enzymes and fermentation bacteria. It was found in our experiment that, during the anaerobic fermentation Lfy silage maize hybrids produced more biogas (640 liter per 1000 g dry matter) than conventional hybrids (606 liter per 1000 g dry matter) (Table 4).

The difference was statistically significant. In both years the lowest biogas production was recorded for the same hybrid (Mv NK 333, 546 l per 1000 g dry matter), while a Lfy hybrid (Mv Massil, 659 l per 1000 g dry matter) produced the greatest biogas yield. The best quality biogas contains 60% methane. There were no significant differences between the methane content of Lfy and non-Lfy hybrids tested in the experiment. The process of outgassing took three weeks and the rate of outgassing was 87%, averaged over years and hybrids (Figure 3).

Summary and Conclusions

The development of leafy silage maize hybrids was an evolution of thought and germplasm that has led to an improvement in silage maize management. The use of Lfy hybrids (in different maturity groups) with their significantly greater canopy structure should be a good option for silage growers. Silage maize breeders looking for inbred parental lines which produce hybrids with good germination and early season growth under both optimal and suboptimal conditions; rapid spring development to make a closed canopy sooner; large plants having both large leaves and extra leaves; soft kernels at maturity with less of the hard vitreous starch and more soft starch; flexible rather than rigid stalks; good mature leaf disease resistance to keep the plant green and produc-

Table 3 - Comparative studies of some quality traits of the leaves above the ears of 2 Lfy and 2 conventional maize hybrids (Martonvásár, 2010)

Morphological traits	Limasil (Lfy)	Mv NK 333 (conventional)	Kámasil (Lfy)	Maxima (conventional)	$LSD_{5\%}$
	FAO 300		FAO 500		
1 Weight of fresh leaves (g)	94.00	96.13	112.63	83.88	14.61
2 Dry matter (%)	32.03	30.43	34.92	26.65	3.62
3 Lignin (%)	6.77	6.95	6.27	6.34	0.47
4 WSC (%)*	6.51	7.06	5.25	5.16	1.25
5 WSC (kg/ha)	430.20	474.05	414.62	302.77	104.36
6 DIGOM (%)*	56.57	53.43	57.02	56.13	1.73
7 UL DIGOM* (t/ha)	3.72	3.60	4.49	3.30	0.61

*WSC: water soluble carbohydrates (incl. simple sugars); DIGOM: total digestible organic matter (%); UL DIGOM: total digestible organic matter of the upper leaves above the ear (t/ha)

Table 4 – Quality traits of the silage hybrids related to biogas production (2009-2010)

Hybrid type	DM (%)*	Measured dry matter components				Biogas (l/kg) DM	Methane (%)
		Starch**	Lignin**	NDF**	WSC**		
4 non-Lfy hybrids	27.77	34.86	4.27	53.62	5.50	606	60.10
4 Lfy hybrids	29.14	36.40	4.14	54.52	5.54	640	60.80
LSD5%	0.43					12.00	0.84

* % of fresh weight; ** % of dry matter

tive; early flowering and a longer grain filling period can also find these characteristics in Lfy genotypes.

It was found in the experiments that leafy hybrids have lower ear attachment height, resulting in a lower centre of gravity and allowing greater stalk flexibility, and a significantly greater photosynthetic leaf area to provide the ear with carbohydrates and higher soluble sugar levels in the leaves. The higher number of leaves above ear means that they can shade each other more than those of non-Lfy hybrids. As a consequence, choosing the optimal plant density is a very important factor in the production of Lfy hybrids.

Leafy hybrids have a larger number of younger leaves above the ear – with lower lignin content - during the harvesting period, at the end of waxen ripeness, when the total dry matter of the plant is about 30-35%. This allows high sugar content in both the stalk and the leaf, helping fermentation and giving a sweet fragrance to the silage. According to the farmers' experiences, feeding animals with this type of Lfy silage increases the cow's intake, so milk production will be higher.

The results of experiments on Lfy hybrids revealed that hybrids of this type could also find an application in the production of renewable energy (biogas).

Based on cooperation between the Agricultural Research Institute of the Hungarian Academy of Sciences and the Canadian company Glenn Seed Ltd, a total of five joint Lfy hybrids have been registered in Hungary so far and these hybrids are on the European Union's Variety List.

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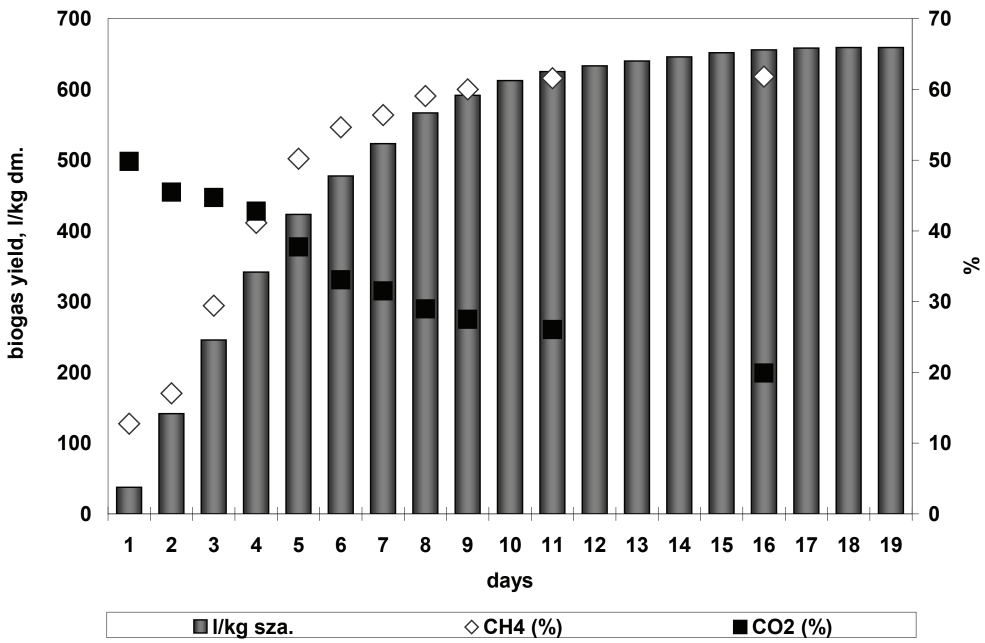

Figure 3 - Specific biogas yield from the maize hybrid Mv Massil averaged over the years 2009-2010

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