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GENETIC VARIATION FOR ORGANIC MATTER AND CELL WALL DIGESTIBILITY IN SILAGE MAIZE. LESSONS FROM A 34-YEAR LONG EXPERIMENT WITH SHEEP IN DIGESTIBILITY CRATES

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ABSTRACT - In long term experiment, the *in vivo* feeding values of silage maize was investigated in 2383 mini-silos with sheep in digestibility crates. Among the 478 investigated hybrids, 178 were experimental hybrids, including 45 *bm3* hybrids, and 297 were registered hybrids. Most hybrids (403) were early hybrids (FAO 170 to FAO 350). Among the normal hybrids (excluding *bm3*), the cell wall digestibility (estimated as NDF digestibility, or NDFD) nearly doubled from 35.9 to 60.4%, and similar values were observed for the sub-sample of early registered hybrids (39.3 to 58.7%). The range of variation was lower for organic matter digestibility (OMD), equal to 11%, due to the diluting effect of grain, which is a highly digestible portion. The OMD variation of early registered hybrids is mostly attributable to NDFD variation (r^2 = 0.59), and to grain content variation (r^2 = 0.10). A similar result was observed when all hybrids were considered simultaneously. A significant drift of hybrids towards lower *in vivo* digestibility values was observed between 1958 and 2002. Only 18% of hybrids registered before 1989 had a lower NDFD than Helix (NDFD = 47.6%), but the results were 45% when registered between 1989 and 1998, and 70% when registered after 1999. The average range of NDFD improvement brought by the *bm3* gene was equal to 8.7%, and ranged from 0.9 to 17.9%, with a tendency to a higher efficiency in hybrids of lower NDFD. The correlative average OMD improvement was equal to 3.5%, corresponding to an increase in energy value equivalent to the daily energy needed to produce about 2.5 kg milk in cow eating 15 DM silage maize. The search for highly digestible silage maize will require an investigation of old or forgotten genetic resources not currently used in either grain or silage maize breeding. Because there is obviously a considerable gap for agronomic traits between these resources and modern hybrids, specific strategies of introgressing feeding value traits in elite germplasm have to be considered. Moreover, improvement of silage maize NDFD will simultaneously have a beneficial effect on maize intake, which is also a major nutritional factor in cattle feeding.

KEY WORDS: Maize; Corn; Silage; Digestibility; Ingestibility; Cell wall; Genetic improvement; *Brown midrib; bm3*.

INTRODUCTION

Grasslands and savannas cover about 20% of the earth's landscape (JACOBS *et al.*, 1999), of which forage grasses comprise a major source of nutrients for wild or domesticated ruminants and herbivores. Although forages contain almost the same amount of gross energy as cereal grains per unit of dry matter, the energy value of forage grasses is lower and much more variable, ranging approximately from 33% (wheat straw) to 70 (silage maize) or 80% (leafy ryegrass) of maize grain value. The difference in energy values between grain and forage results from the high content of cell wall in forage plants, and to the limited digestion by herbivorous animals of this fiber fraction by micro-organisms in the digestive tracts of animals. Lignification of cell walls is the major factor limiting the degradability of grasses in the rumen and/or large intestines of herbivores. However, large inter-species variation in organic matter or cell wall digestibility were documented, and significant genetic variation was also demonstrated within species (review in BARRIÈRE *et al.*, 2003a).

Among grasses, maize is a model plant for genetic and genomic research projects, but maize is also a major forage crop. Today, about 4.6 Mha of maize are grown for silage in the new European Union that includes 25 countries. Silage maize provides roughage with a high energy content, and its low protein content is easily corrected in the dairy cow diets with cattle cakes. Specific maize breeding for silage traits is quite recent. For a long time breeders relied on the believe that breeding grain

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maize was also convenient for silage use. Nowadays, maize registered hybrids used for silage are mainly genotypes selected from whole plant experiments, but their grain maize based germplasm remains sizeable. In most European countries, grain and silage markets are indeed of similar extent, and breeding programs are significantly inter-related, often for budgetary reasons.

In most cases, for practical and cost reasons, comparisons of silage maize's *in vivo* digestibility were performed using sheep, even if dairy cows, steers, or young bulls are the main consumers. However, from former works of CHENOST and MARTIN-ROSSET (1985), AERTS *et al.* (1984), and MORAN *et al.* (1988), milking cows and sheep fed the same maize silages gave the same ranking of maize digestibility. Silage maize is probably a little better digested by cows than by sheep, but average reported differences were lower than 5%. A decrease of maize cell wall digestibility is observed when negative associative digestibilities between cellulose and starch occur in rumen of cattle fed a content too high in highly digestible starch (EL-SHAZY *et al.*, 1961; FAVERDIN *et al.*, 1987; BARRIÈRE and EMILE, 1990). Based on different European experiments (review in BARRIÈRE *et al.*, 2003a), the genetic variation in the digestibility value of maize silage measured in sheep was also proven to have consequences on young bull or dairy cow performances, even if maize was not the only constituent of the diet. All other factors being equal, when comparing hybrids with poor or good cell wall digestibility in dairy cows, fat corrected milk (FCM) yields differed from 1 to 3 kg among hybrids. The protein contents in milk were also equal or higher in hybrids that allowed greater milk yields. In a similar way, differences in average daily gains of young bulls reached 100 g per day among hybrids. Organic matter and cell wall digestibilities estimated from sheep experiments are thus the most relevant way to investigate the genetic variation for these traits in forage maize, even if breeding maize for silage use must obviously be based on correlated *in vitro* traits, or on NIRS (near infrared reflectance spectroscopy) calibrations.

Most often, feeding value measurements in sheep were been devoted to studies of whole plant digestibility. Only a few studies have investigated cell wall digestibility, whereas this latter trait is now proven as the most relevant one. The first digestion trials in Europe with ensiled forage maize were those of DIJKTRA and BECKER (1960), quoted in DEINUM *et al.* (1984), in the Netherlands, and those of DEMARQUILLY (1969), and ANDRIEU and DEMARQUILLY (1974), in France. From 25 measurements reported by DEINUM *et al.* (1984), organic matter digestibility (OMD) of silage maize had an average value of 72.8%. The average NDF *in vivo* digestibility (NDFD) of hybrids was 52.7%, and ranged from 47.5 to 57.1%. ANDRIEU and DEMARQUILLY (1988) later reported an average OMD in maize silage equal to 71% that could reach 74%, according to cropping conditions, maturity and/or grain content. The NDFD of hybrids involved in this French study was later estimated to 53.1%, and ranged from 49.8 to 54.8% (BARRIÈRE and ARGILLIER, 1997). From 50 maize silages cropped between 1971 and 1993, DE BOEVER *et al.* (1988, 1997) reported average OMD values of 74.7%. The average NDFD value of these hybrids, computed from crude fiber values, was 61.4%, and ranged from 56.1 and 68.8% (DE BOEVER unpublished data, quoted in BARRIÈRE *et al.*, 2003a.). However, most of these forage maize *in vivo* reference values, estimated more than 30 years ago, were based on a limited number of early genotypes with good feeding value such as Fronica, Circé, LG11, (but not Eta Ipho), in the Netherlands, and Inra258, Funk245, Dekalb204, LG11, in France, which are no longer representative of the presently cultivated hybrids.

In numerous feeding value experiments with silage maize, *brown-midrib3 (bm3)* hybrids were used as relevant controls for high digestibility values. The *brown-midrib* gene, described by EMERSON (1935), was first proven to have an effect on lignin content by KUC and NELSON (1964), and the improved digestibility of *bm3* plants in cattle was first described by BARNES *et al.* (1971). Later, GRAND *et al.* (1985) established the quasi-lack of OMT (O-methyl transferase) activity in *bm3* plants. VIGNOLS *et al.* (1995) showed that a large part of intron 2 was deleted in the *bm3* mutant OMT gene. Since 1971, numerous works showed the higher digestibility of *bm3* maize plants, either from *in vivo* or *in vitro* experiments (CHERNEY *et al.*, 1991; BARRIÈRE *et al.*, 2003a). Considering feeding experiment with sheep, MULLER *et al.* (1972), GALLAIS *et al.* (1980) and BARRIÈRE *et al.* (1985, 1998) demonstrated an improvement of crude fiber digestibility by 10 to 20%. In the experiment of DEINUM *et al.* (1984), NDFD of one *bm3* hybrid was as high as 65.1%.

Many measurements of silage maize whole plant and cell wall digestibility were made on a large genetic basis at INRA Lusignan (Vienne, France) with sheep in digestibility crates. The first report was given by BARRIÈRE *et al.* (1992). This long-term experiment was set up by A. GALLAIS in 1969, and experi-

ments were performed every year up to the 2002 harvest. This paper reports information and conclusions about genetic variation of *in vivo* OMD and NDFD in silage maize, effect of the registration year, and effect of the *bm3* gene from a genetic basis never previously or elsewhere investigated, and gathering maize hybrids representative of about 45 years of European breeding.

MATERIALS AND METHODS

The feeding value measurements in sheep were performed at INRA Lusignan between 1969 to 2002. Average rainfall and heat units (basis 6) during these three decades are given in Table 1. Maize was cropped at 95,000 plants/ha, except very late hybrids cropped at 75 to 85,000 plants/ha. Row spacing was 0.75 m. Irrigation was given since 1975 two or three times at 30 mm to prevent summer water stress. Each year, two plots (or replicates), each of them measuring 150 m^2, were cropped for each studied hybrid. In a few cases only one plot was cropped because of seed limiting quantities, or three or four plots were cropped to get earlier a more acute value on one genotype. During September, the maize hybrids were harvested at the (hard)-dough stage to yield silage with a DM (dry matter) content between 30 and 35%. DM yield of hybrids was estimated at harvest from mini-silo fresh weights and DM content was estimated from about 1 kg samples. Each maize plot was then ensiled, according to standard farming practices, in one cylindrical mini-silo (1.60 m height, 1.45 m diameter) made of an airtight plastic bag (150 µ) set in a wire fencing metal frame (TRAINEAU, 1991).

At least two months after harvest, each silage was fed twice daily to six Texel wethers, housed individually in digestibility crates. Only nitrogen (1.5% urea), sodium bicarbonate (20 g), minerals and vitamins were added to balance the maize diet. Until 1995, sheep were individually fed *ad libitum*, with approximately 10% refusals, and their average intake was 48 $g/kg^{0.75}$. Since 1996, in order to simplify the experimental management, feeding was adjusted to the maintenance requirement of each animal, estimated to be equal to 40 $g/kg^{0.75}$, and given according to sheep metabolic weight. The effect of feeding level on digestibility is complex, but mainly for animals fed 3 to 5 times the maintenance level. During the 1969 - 1995 period of this experiment, sheep fed *ad libitum* ingested an average of only 1.2 times the maintenance level. It was then considered that this effect could be confused with the year effect, all the more as observed genotype x feeding level interactions were non significant ($P > 10\%$) for digestibility traits. For each of the mini-silos, data were collected over five days, after a seven day pre-experimental period. *In vivo* digestibilities of organic matter (OMD) and cell wall were determined from contents in offered forage and in faeces, according to JARRIGE (1988). Cell wall content was first estimated as crude fiber content (Weende method; HENNEBERG and STOHMANN, 1859), because this trait was the most widely used at the beginning of this long-term experiment. Since 1996, it was additionally estimated as NDF (neutral detergent fiber, GOERING and VAN SOEST, 1971). Values of NDF content and NDF digestibility (NDFD) were then computed for silage studied before 1995 using linear regressions between NDF and crude fiber content, or NDFD and crude fiber digestibility, respectively. These regressions were built over the 7 years with both the measurements, and had r^2 values higher than 0.86.

During the entire experiment, crude protein (Kjeldahl nitrogen × 6.25) content of the silage was estimated from wet chemistry measurements. Grain content was estimated for each plot at harvest from a sample of 15 plants up to 1995. Since 1996, starch content of a fresh sample of each plot was estimated with the Ewers method (AFNOR, 1981, EEC ISO 10520.2), a method that is very less time consuming. Grain content was then computed from starch content, and conversely, using the ANDRIEU *et al.* (1993) regression.

2383 mini-silos were studied between 1969 and 2002, corresponding to 478 hybrids. Among these hybrids, 178 were experimental hybrids, including 45 *bm3* hybrids, and 297 were registered hybrids, representative of all seed companies present on the French and North European markets. A few registered hybrids were tested with a confidentiality agreement, and were named Rh. Most hybrids (403) were very early, early or medium early flint x dent hybrids, and medium early dent hybrids (FAO 170 to FAO 350, "early" hybrids), including 248 registered hybrids. Only 43 hybrids were medium late and 32 were late (FAO 400 and 500, "late" hybrids). Some well-known or control hybrids (Inra258, Inra260, Inra260 *bm3*, LG11, LG11 *bm3*, Dea, Adonis, Adonis *bm3*, Dk265, Rh162, Lh74 x F271) were partly successively repeated each year, allowing multi-year mean estimates for each genotype.

Basic data for variance analyses were average (sheep) observations for each mini-silo. Variance analysis were done according to a standard fixed statistical model, with a year effect, a replicate nested in year effect, a genotype effect, and a genotype x year interaction effect. Similar variance analyses were investigated over the whole 2383 mini-silos, and over different sub-sample of hybrids (normal hybrids, early hybrids, early registered hybrids, early normal hybrids, late registered hybrids). Genotype means (and ranges) were estimates from a fixed model without an interaction effect over the whole 2383 data. Because of the data disequilibria and lack of observations of many combinations, genotype x year interactions could be imperfectly estimated when based on a large number of hybrids. A variance analysis was then investigated in the sub-sample of 27 normal early hybrids studied during at least 5 years (564 mini-silos). A specific variance analysis and mean estimate were devoted to the sub-sample of 31 genotypes and their isogenic *bm3* counterparts, over 24 years and 554 mini-silos. Genetic variation in early hybrids across eras of breeding was based on three periods of registration, 1958 to 1988, 1989 to 1998, and from 1999 to 2002. Year 1989 was previously proven as an important switch in digestibility values of hybrids (BARRIÈRE and ARGILLIER, 1997), and 1999 was the first year in France with an *in vitro* digestibility value used as a registration trait. Genotypic variances were estimated according to the Φ_g genotypic meansquare (MS), similar to a variance in a random model (DAGNELIE, 1973; HENDERSON, 1975, model III without genotype x year interaction). They were computed as $\Phi_g = (MS_g - MS_r) / n_g$ where MS_g and MS_r are genotypic and residual MS, respectively, and n_g the average number of genotype observations. Φ_g is the "variance" of the fixed effects. Genotypic correlation between two traits was derived from their genotypic mean product. These genotypic correlations can also be considered as the correlations between fixed effects of two traits. In comparison to phenotypic correlations, the interest of such a formulation is to suppress the effects of environmental variation and covariation. Modli (KOBILINSKY, 1983) and Amance (BACHACOU *et al.*, 1981) softwares were used for statistical computation.

RESULTS AND DISCUSSION

Genetic variation for digestibility and agronomic traits

The INRA research center of Lusignan is situated 46°26 North latitude and 0°08 East longitude, in a place well suited for maize cropping of hybrids with an earliness ranging between FAO 150 and FAO 400. During the three decades between 1969 and 2002, no rainfall changes were observed during the maize cropping season, whereas an increase in heats-units basis 6 °C was observed, nearing 150 °C heat-units, thought to be more a sign of the global warming occurrence (Table 1). Average dry matter content of all investigated silages was 34.5%, with an average crude protein content equal to 8.0%, and an average grain content equal to 43.2%, corresponding to a starch content equal to 28.6%.

OMD and NDFD MS nearly doubled when *brown-midrib* genotypes were included in the variance analyses (Table 2), whereas NDF and grain content MS, or yield MS, were about similar in both analysis. In the sub-sample of early registered hybrids, OMD and NDFD were also highly significant, with MS having values close to those observed among all normal hybrids, indicating a large range of variation for digestibility traits in the set of early hybrids. Variation for investigated traits was lower in late registered hybrids than in earlier ones, except for grain content, but the number of observed hybrids was also lower. Residual MS were important for NDFD, as previously observed (BARRIÈRE *et al.*, 2003a), due to cumulative imprecisions in NDF measurements both in offered silage and faeces.

TABLE 1 - *Average meteorological conditions during the maize cropping and sheep feeding 1969 - 2002 experiments.*

Years	Rainfall (mm) July 01st August 31st	Rainfall (mm) April 20th Sept 20th	Heat-Units basis 6°C April 20th Sept 20th
1969-2002	98	295	1639
1969-1980	91	306	1555
1981-1990	97	292	1592
1991-2002	101	301	1724

TABLE 2 - *Variance analysis for OMD* (in vivo *organic matter digestibility*), *NDFD* (in vivo *NDF digestibility*), *NDF (neutral detergent fiber) and grain contents, and yield in maize silage.*

Hybrid type	df	OMD %	NDFD %	NDF %	Grain %	Yield t/ha
All hybrids						
Genotype MS	477	21.7 **	90.2 **	24.2 **	71.0 **	18.7 **
Genotype x year MS	189	3.2 ns	18.0 ns	7.3 *	27.4 **	3.9 **
Residual MS	1644	3.0	17.5	6.1	14.5	2.5
All normal hybrids						
Genotype MS	424	14.7 **	47.4 **	22.8 **	66.0 **	13.7 **
Genotype x year MS	167	3.1 ns	18.8 ns	7.5 *	27.6 **	3.8 **
Residual MS	1404	3.0	17.7	6.1	13.8	2.6
Early registered hybrids						
Genotype MS	247	12.3 **	49.5 **	20.3 **	41.2 **	13.2 **
Genotype x year MS	117	3.2 ns	19.0 ns	6.9 ns	28.1 **	3.8 **
Residual MS	1012	2.9	17.3	5.9	12.0	2.5
Late registered hybrids						
Genotype MS	50	6.8 **	33.4 **	14.1 **	42.8 **	5.2 **
Genotype x year MS	23	2.6 ns	17.9 ns	5.8 ns	28.1 **	4.6 *
Residual MS	123	2.3	14.2	5.0	12.6	2.6
Early normal hybrids (year nbr ≥ 5)						
Genotype MS	26	33.0 **	136.9 **	28.4 **	60.6 **	35.0 **
Genotype x year MS	133	4.0 **	21.5 ns	9.9 **	29.9 **	5.0 **
Residual MS	337	2.6	16.4	5.6	9.0	1.2

df = degree of freedom; MS = mean-square.
* and ** = Significant at $P < 0.05$ and $P < 0.001$, respectively; ns = non significant at $P > 0.10$.

TABLE 3 - In vivo *mean and range of variation for OMD and NDFD in maize silage (OMD is* in vivo *organic matter digestibility, NDFD is* in vivo *NDF digestibility, and NDF is neutral detergent fiber).*

		OMD			NDFD		
Hybrid type	hybrid number	mean	mini	maxi	mean	mini	maxi
All hybrids	478	70.0	61.3	76.9	49.6	32.9	65.6
All normal hybrids	425	69.6	61.3	76.0	48.6	32.9	60.4
All *bm3* hybrids	45	73.6	68.1	76.9	58.6	52.3	65.6
Early normal hybrids	358	69.9	62.9	76.0	48.8	35.9	60.4
Early registered hybrids	248	69.8	65.4	74.4	48.2	39.3	58.7
Early *bm3* hybrids	37	73.8	68.6	76.9	58.4	52.3	63.8
Late normal hybrids	67	67.8	61.3	71.7	47.5	32.9	58.1
Late registered hybrids	53	67.6	61.3	71.7	46.6	32.9	58.1
Late *bm3* hybrids	8	72.8	68.1	75.1	59.5	55.3	65.6

TABLE 4 - *Genotypic correlations between traits related to* in vivo *feeding value in maize hybrids (OMD is* in vivo *organic matter digestibility, NDFD is* in vivo *NDF digestibility, and NDF is neutral detergent fiber, Φ_g coefficient is similar to a variance in a random model and estimated as $\Phi_g = (MS_g - MS_r) / n_g$).*

	OMD	NDFD	Grain content	NDF content	Φ_g
Normal hybrids					
OMD	–				2.55
NDFD	0.77	–			6.45
Grain content	0.54	0.10	–		11.07
NDF content	–0.72	–0.06	–0.73	–	3.60
Yield	–0.53	–0.52	–0.12	0.27	2.40
Early registered hybrids					
OMD	–				1.74
NDFD	0.76	–			5.99
Grain content	0.32	–0.16	–		5.13
NDF content	–0.55	0.10	–0.63	–	2.68
Yield	–0.55	–0.56	0.13	0.18	2.00

Among the normal maize hybrids (excluding *brown-midrib* ones), the NDFD nearly doubled from 32.9 to 60.4% (Table 3), and it was nearly similar for the sub-sample of early registered hybrids for which the NDFD went from 39.3 to 58.7%. Variation was lower for OMD, with an average range equal to 11% in the same sub-sample, due to the diluting effect of grain, which is a highly digestible component. Late hybrids had average OMD and NDFD slightly, yet significantly, lower than early hybrids, and a few late hybrids also had a much lower cell wall digestibility than early hybrids. Whereas very few normal hybrids had seemingly a high NDFD, no normal hybrid reached the upper value observed in *bm3* hybrids, and above all, the lowest values observed in *bm3* hybrids were much higher than the lowest value observed in normal hybrids. A few experimental normal hybrids also had a higher NDFD than the highest observed in registered hybrids. An example is the three-way hybrid (W94129 x F7019) x F4, which had a NDFD equal to 59.7%. W94129 is a dent line bred by J. Coors in the Wisconsin Quality Synthetic (WQS). F7019 is a dent line bred at INRA Lusignan for cell wall digestibility, and F4 is an old very early flint INRA line, having a very high cell wall digestibility (MÉCHIN *et al.*, 2000; FONTAINE *et al.*, 2003).

Genotype x year interactions were not signifi-

cant for digestibility traits (OMD and NDFD), but were significant for yield, grain and NDF contents, except for NDF content in early and late registered hybrids. When considering only the sub-sample of 27 hybrids studied over 5 or more years, the genotype effect for NDFD was highly significant, whereas the NDFD genotype x year interaction was not significant (Table 2). Conversely, a very high genotype x year interaction was observed for grain content, and, to a lower extent, for NDF content. As a consequence, OMD genotype x year interaction was significant, but the interaction MS was about 10 times lower than the genotype MS. A similar result was previously obtained from a specific experiment in 14 locations, showing that *in vivo* crude fiber digestibility genotype x environment interaction was non-significant, whereas the location and genotype main effects were highly significant (ARGILLIER *et al.*, 1997). Maize breeders are able to improve maize feeding value from an estimate of cell wall digestibility, after maize cropping in a limited number of locations and/or years, provided the locations are well chosen, and provided a relevant *in vitro* criterion is available.

The study of genotypic correlations (Table 4) showed that OMD was related greatly to NDFD (r^2 = 0.59) both for all hybrids and the early registered ones, and actually little to grain content, especially for early registered hybrids (r^2 = 0.10). The correlation between NDF content and NDFD was close to zero, evidencing that no significant relationship existed between the cell wall digestibility and the cell wall content when maize plants were harvested at a similar maturity stage. Consequently, for a given grain (starch) content, OMD was related almost only to NDFD. The genetic progress in feeding value was thus directly related to NDFD improvement. Breeding efficiency will also be higher if based on NDFD, because OMD gathered confusing effects of NDFD and grain content. Both NDFD and OMD were negatively correlated with whole plant DM yield (r close to 0.55). However, this correlation is not likely to prevent breeding hybrids with both high digestibility and agronomic values.

The direct use in breeding programs of a trait such as NDFD is obviously not possible, and plant breeders must use a correlated *in vitro* trait. However, it was also possible to develop a NIRS calibration of this value. Based on 1672 spectra corresponding to 866 mini-silos and sheep references over 6 years (1997 to 2002), the coefficient of determination and the standard error of cross validation of the developed calibration for NDFD were 0.64 and 4.14, respectively. Similarly, the coefficient of determination and the standard error of cross validation were 0.63 and 1.89 for OMD, respectively. These values can be compared to the "reference" value of the enzymatic solubility calibration developed in CRA Libramont from 1631 spectra, whose coefficient of determination and standard error of cross validation were 0.84 and 2.23, respectively. The quality of *in vivo* traits prediction is then little lower, but the measures of digestibility value in animals are also less repeatable than *in vitro* solubility measurements.

However, the accuracy of this NIRS prediction of OMD was similar or little better than the currently used prediction based enzymatic solubility. From the results of ZIMMER *et al.* (1980, quoted in ZIMMER *et al.*, 1990), GIVENS *et al.* (1995), DE BOEVER *et al.* (1997, 1999) and ANDRIEU *et al.* (1995), *in vitro* estimates of whole plant digestibility explained a portion ranging between 50 and 60% of the variation observed in cattle. But, while relationships OMD and IVDMD (*in vitro* dry matter digestibility) were significantly investigated, very few papers have reported data on intra-specific relationships between *in vitro* and *in vivo* cell wall digestibility estimates in maize. From the experiments of ARGILLIER *et al.* (1998) and DE BOEVER (unpublished data, quoted in BARRIÈRE *et al.*, 2003a), cell wall digestibility computed traits based on enzymatic solubility or TILLEY and TERRY (1964) degradability explained from 30 to 45% of *in vivo* crude fiber digestibility. From a preliminary study (BARRIÈRE *et al.*, 2003a) in a sub-sample of 4 years of experiment and 165 maize hybrids (including *bm3*) from the 2383 mini-silos, IVDMD explained 55% of OMD variation, and DINAGZ explained 56% of NDFD variation (DINAGZ is the *in vitro* digestibility of the "non starch (ST), non soluble carbohydrates (SC) and non crude protein (CP) part", and is computed as DINAGZ = 100 x (IVDMD – ST – SC – CP) / (100 – ST – SC – CP), according to ARGILLIER *et al.* (1995a) and BARRIÈRE *et al.* (2003a).

Voluntary intake is simultaneously with plant digestibility a primary nutritional factor controlling animal production. Dry matter content of the silage is an important factor of intake variation, and optimum water content between 32 and 37% has been established, allowing a good conservation, a good palatability and a good intake of maize silages. However, for a given dry-matter content, ingestibility is the plant trait, also subject to genetic variation, estimated in animals as intake. Results obtained

with the early hybrid Dk265 illustrated clearly the specificity of the ingestibility trait in maize (BARRIÈRE *et al.*, 2004a). Few data on the relationships between genetic variation for digestibility and genetic variation for ingestibility are available, mostly due to the near impossibility for plant breeders to work with cattle. However, 50 to 77% of the intake variation was explained by NDFD (BARRIÈRE *et al.*, 2003b), as this trait is also very likely related to the rate of particle degradation. However, the ingestibility and/or the filling capacity of corn forage probably depends on another genetic traits of the corn plant related to its mechanical resistance to chewing by cows, traits that should be considered as (partly) independent from NDFD (BARRIÈRE *et al.*, 2004a).

Effect of registration year on digestibility traits

A significant drift of hybrids towards lower *in vivo* digestibility values has been observed in the last two to three decades, and hybrids registered before 1989 appeared to be more digestible than hybrids registered between 1989 and 1994 (BARRIÈRE and ARGILLIER, 1997), or from preliminary analysis, between 1989 and 2000 (BARRIÈRE *et al.*, 2003a). Digestibility traits were indeed not considered in France for forage maize registration until 1998. Studies of digestibility values in early hybrids registered during the three eras 1958-1988, 1989-1998, and 1999-2002 showed a continual tendency to a lower feeding value in the most recent hybrids (Table 5). When compared to the early hybrid Dk265 (OMD = 71.8%, NDFD = 51.4%), 25% of early hybrids registered before 1989 had a higher OMD than Dk265, but they were only 7% of the hybrids registered between 1989 and 1998, and none after 1998 (38, 10, and 7% of hybrids for NDFD, respectively). Conversely, only 18% of hybrids registered before 1989 had a lower NDFD than Helix (OMD = 69.1%, NDFD = 47.6%), but they were 45% when registered between 1989 and 1998, and 70% when registered after 1998 (17, 28 and 57% of hybrids for OMD, respectively). For cows eating 15 kg DM silage maize per day, and based on energy values, Dea (registered in 1980) allowed the yield of 0.8 kg milk less than Inra258 (registered in 1958), whereas Anjou285 (registered in 1994) allowed 2.6 kg milk yield less than Inra258, and the poorest hybrid registered in 2002 allowed 3.9 kg milk yield less than Inra258. However, Rh383, registered in 2001, allowed a similar milk yield as Dea, with a DM yield higher by about 4 t/ha. Because the effect of registration period on grain content was non significant, even if means showed a tendency to a slight increase, the decrease in digestibility was directly related to a decrease in cell wall digestibility, with an average decrease close to 5 percentage points. The use of an *in vitro* digestibility trait for hybrid registration since 1999 does not appeared efficient to modify this evolution. However, four years was probably too short a period for a tendency reversal, all the more so as germplasm is deeply oriented in grain breeding and registration control hybrids also originated from grain breeding and, for a significant part of all, are of mediocre digestibility.

Considering agronomic traits, highly significant improvements in maize yield, yield stability, stalk standability, stalk rot resistance and stay-green have been achieved in the last five decades in Europe (DERIEUX *et al.*, 1987; BARRIÈRE *et al.*, 1987), and in the last century in the USA (RUSSEL, 1984; TROYER, 1999, 2002). In forage maize (BARRIÈRE *et al.*, 2003a), the whole plant yield progress was found to be close to 0.17 t/ha/year for hybrids registered in France between 1986 (the first year with registration after forage maize official trials) and 2000, including agronomic and genetic improvements. Based on average data between 1958 and 2002, the average genetic improvement in whole plant yield appeared here close to 0.10 t/ha.year in the 1958 - 1988 period, and close to 0.14 t/ha.year between 1989 and 2002. In the period before 1986, whole plant genetic progress were expected to be less important in hybrids mostly bred for grain yield. However, a similar trend towards an increase in the rate of genetic improvement was also observed in maize grain yield. The average genetic improvement was close 0.117 t/ha.year during the 1980 - 1990 period, but it was close to 0.130 t/ha.year between 1990 and 2000 (GALLAIS, 2002 and unpublished data). An almost similar increase in both grain and stover parts could be hypothesized, because there were only a tendency to a little (but non significant) increase in grain content in modern hybrids compared to older ones (Table 5). In the USA, LAUER *et al.* (2001) highlighted an annual rate of forage yield increase of 0.13 to 0.16 t/ha since 1930. But they did not find any change of the *in vitro* digestibility of the whole plant, nor of the cell wall digestibility, whereas major improvement in stalk standability, and in stalk rot resistance, were achieved during the same period. The discrepancy in digestibility evolution between European and US results could be due to a different evolution of hybrid germplasm in Europe and in the USA. The maize improvement in the US was very

TABLE 5 - *Variance analysis for OMD, NDFD, NDF and grain contents, and yield in maize silages according to breeding eras (OMD is* in vivo *organic matter digestibility, NDFD is* in vivo *NDF digestibility, and NDF is neutral detergent fiber).*

Early registered hybrids		OMD %	NDFD %	NDF %	Grain %	Yield t/ha
	df					
Registration period MS	2	209.5 **	595.5 **	97.2 **	14.3 ns	240.0 **
Genotype / registration period MS	243	10.5 **	45.4 **	19.7 **	41.2 **	11.5 **
Genotype / regist. period x year MS	339	4.3 **	25.1 **	8.1 **	21.4 **	5.0 **
Residual MS	783	2.4	14.2	5.1	10.5	1.6
	nb					
mean registered 1958 – 1988	65	70.7	50.3	40.2	43.2	13.8
mean registered 1989 – 1998	137	69.7	48.0	40.6	44.7	16.3
mean registered 1999 – 2002	44	69.0	45.7	39.9	45.1	18.1

df = degree of freedom; nb = number of hybrids; MS = mean-square.
** = Significant at P < 0.001; ns = non significant at P > 0.10.

TABLE 6 - *Comparison of normal and* bm3 *hybrids for digestibility and agronomic traits (OMD is* in vivo *organic matter digestibility, NDFD is* in vivo *NDF digestibility, and NDF is neutral detergent fiber).*

	OMD (%)		NDFD (%)		Yield (t/ha)		Grain (%)	
Normal / *bm3* MS	1420.7 **		8826.4 **		329.0 **		357.7 **	
Genotype MS	28.2 **		90.1 **		25.4 **		117.0 **	
Genotype x normal / *bm3* MS	4.2 ns		21.2 ns		6.2 **		24.8 ns	
Genotype x year MS	1.8 ns		8.7 ns		4.6 *		14.6 ns	
Residual MS	3.2		17.1		2.2		16.8	
	normal	*bm3*	normal	*bm3*	normal	*bm3*	normal	*bm3*
Mean 31 hybrids	70.0	73.5	49.4	58.1	14.3	12.4	43.8	41.8
Mini	66.0	67.2	43.1	50.9	7.8	4.7	28.2	25.5
Maxi	73.5	76.3	58.6	64.2	19.8	16.6	55.1	53.5
registered in								
Inra258 1958	72.2	74.5	53.8	60.1	11.7	11.2	44.0	46.4
LG11 1970	71.5	74.3	50.8	60.4	12.7	11.6	45.5	45.3
Adonis 1984	70.4	73.9	48.7	56.2	16.2	13.5	45.5	42.2
Dk265 1987	71.4	75.4	50.0	61.5	13.7	12.1	45.9	42.5
Rh162 1990	67.4	72.0	43.1	54.1	17.1	14.8	44.8	43.0
Helix 1993	68.6	74.9	46.0	58.2	15.9	13.2	44.8	46.5

. **, *, ns = Significant at P < 0.001 and 0.01 or non significant at P >0.05, respectively.

likely carried out without major germplasm changes, and continuously based on the Reid and Lancaster groups. Conversely, dent lines in modern European hybrids are now related more to Iodent and Reid origins than were old early dent lines used in Europe, with higher cell wall digestibility. Moreover, old European flint lines of high cell wall digestibility such as F7 are now no longer involved in the modern flint germplasm, due to their low combining ability values for yield, and their stalk rot or stalk lodging susceptibility. Some modern early European hybrids are also quasi-dent hybrids.

Comparisons of normal and bm3 *isogenic hybrids*

Genotype effect was significant among the 31 investigated normal and *bm3* hybrids, even if the *bm3* effect was greatly preponderant, except for grain content for which genotype and normal/*bm3* MS were of similar magnitude (Table 6). Genotype x normal/*bm3* interactions were significant only for yield, and not for NDFD, OMD or grain content. Similarly, genotype x year interactions were significant only for yield.

Average grain content was little lower in *bm3* isogenic hybrids compared to normal ones, but some *bm3* hybrids had a similar grain content than their normal isogenic. Average yield was also lower in *bm3* hybrids, with a tendency to a greater decrease in normal hybrids having a higher yield. The correlation between the yield in normal hybrids and the yield decrease in each *bm3* isogenic was indeed negative (r = -0.57). The average OMD improvement between normal and *bm3* hybrids was 3.5%, corresponding to an increase in energy value equal to 0.44 MJ/kg DM (computed according JARRIGE *et al.*, 1989, and equivalent to the daily energy need for about 2.5 kg milk in cows eating 15 kg DM silage maize). The average NDFD improvement between normal and *bm3* hybrids was 8.7%, but the NDFD improvement ranged from 0.9 to 17.9%, showing an effect of the genetic background on the NDFD improvement obtained with the *bm3* gene. The correlation between NDFD values in normal and *bm3* hybrids was only 0.65. Moreover, the correlation between the NDFD in normal hybrids and the NDFD improvement given by the *bm3* gene in each hybrid was -0.48. These results showed a tendency to a lower efficiency of the mutant gene when normal hybrids were of higher cell digestibility. Due to the lower yield of the *bm3* isogenic, the average yield in digestible OM is 0.9 t/ha lower in *bm3* hybrid than in the normal hybrid. However, the interest of the *bm3* mutation is not cancelled out by this fact, because *bm3* silage are also greatly improved in ingestibility, allowing significant reduction in expensive concentrates being given to cattle (review in BARRIÈRE *et al.*, 2003a).

CONCLUSIONS AND PERSPECTIVES

Based on this long-term experiment, the genetic variation in cell wall digestibility of forage maize had a range of about 25% (20% in registered hybrids), with an average cell wall digestibility close to 49%. Moreover, the significant decrease in NDFD values is all the more detrimental to cattle feeding that it also significantly impedes the silage intake. It has thus partly ruined the interest of the outstanding yield improvement. In Europe, most of modern forage maize hybrids are still based on a germplasm bred and/or used in grain maize hybrids over decades. It is then very likely that alleles allowing good digestibility (and also good ingestibility) were largely eliminated during breeding for stalk standability and breakage resistance, or were lost because of genetic drift. The search for highly digestible (and ingestible) maize will require an investigation of old or forgotten genetic resources that are not currently used or that were never used in grain maize breeding. These resources were also neglected because they led to poor-yielding genotypes (lower additive value, and lower heterotic pattern), or because they were susceptible to lodging or stalk rotting. However, according to ARGILLIER (1995b), there is no evidence of an absolute or definitive linkage between these poor agronomic traits and the feeding value in maize. Because there is obviously a considerable gap in agronomic value between these old lines or resources and elite modern lines, specific strategies of introgressing feeding value traits in elite germplasm must be considered. QTL, SNP (single nucleotide polymorphism) or INDEL (insertion – deletion polymorphism) of interest for feeding value have to be identified prior to any breeding effort. This strategy will allow a carefully targeted marker assisted selection (MAS) based on flanking SSR or SNP markers, respectively, with very small modifications of the back-crossed elite lines. Moreover, favorable QTLs can be used as genetic resources and introgressed through MAS in elite lines, even if the underlying determinants are not understood.

Until recently, the precise mechanisms by which lignification reduces degradability remained unclear. Lignins are likely the only component in cell walls resistant to bacterial and fungal degradation in the gut, but their variable association with other cell wall matrix components more or less limits their hydrolysis by digestive enzymes. Agronomic constraints do not allow cell wall digestibility in maize to be improved in breeding only for low lignin content. Lignin structure, cross-linking, particularly via hydroxycinnamates, and lignification patterning were also shown to be major determinants of wall carbohydrates digestibility by micro-organisms (and

probably also to silage ingestibility due to plant resistance to chewing). The understanding of the molecular basis of cell wall biogenesis, lignification and digestibility will be at the forefront of further progress in maize cell wall digestibility and grass lignin pathway understanding (reviews in GRABBER *et al.*, 2004, BARRIÈRE *et al.*, 2004b, and RALPH *et al.*, 2004).

As soon as molecular determinant are evidences, another relevant approach toward breeding maize of higher feeding value is to devise specific genetic resources through genetic engineering of the cell wall biogenesis or lignin pathways. Genetically modified plants could then be directly used for feeding, but transgenesis is also a particularly efficient way to uncover the more relevant targets for a further classical investigation of genetic resources and breeding of normal genotypes. Even if very few papers were devoted to transgenesis in monocotyledons, only PIQUEMAL *et al.* (2002) and CHEN *et al.* (2003) on maize, and HE *et al.* (2003) on fescue, extensive reviews on genetic engineering in the lignin pathway established the more or less efficient antisense or silencing strategies for reducing lignin content and/or modifying lignin structure or cell wall phenolics of plants, in turn increasing cell wall digestibility (BOUDET, 2000, 2003; BOERJAN *et al.*, 2003). If highly variable consequences on lignin content and structure were often observed in altered transgenic plants, these inconsistencies highlight the necessity and the interest to further evaluate the effects of the used promoter, especially because of the interaction with the spatio-temporal deposition in each lignified tissue, and to evaluate the correlative effect on the pathway of each gene down-regulation.

A breeding target must be evidenced clearly before efficient knowledge of genetic resources will become usable for breeding. Reciprocally, for such complex traits, and especially when including ingestibility, a genetic resource can be considered of interest only when it has been proved having a genomic trait related to feeding value, thus allowing its introgressing in the building of a new genitor. Similarly, the improvement of forage cell wall digestibility through genetic engineering supposes an *a priori* knowledge of the role of targeted genes or regulating factors. Reciprocally, an efficient target can be evidenced by the in-depth studies of the biochemical characteristics of cell wall in plants up- or down-regulated for a gene the function of which was not understood. Both *in vivo* measurements of maize cell wall digestibility and biochemical and molecular approaches strengthened the possibility of further significant improvement in maize silage feeding value.

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