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Genetic variation for cell wall degradability in maize inbred lines without and after alkaline pretreatments

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Abstract

Alkali pretreatments were applied at two temperatures on a set of eight maize lines exhibiting a large range of cell wall degradability, which was comprised between 49.4 and 24.9%. The effect of NaOH concentration on cell wall degradation was shown strongly preponderant over the effect of temperature, with significant interactions between alkali concentrations and temperatures. Interactions between lines and NaOH concentration or temperature were weak or non significant. With mild-alkaline 0.02 N pretreatment, cell wall degradation was similarly increased by 36% for all considered lines, without any difference between 22°C or 50°C temperature conditions. With more severe 0.2 N alkaline pretreatment, average cell wall degradation was increased by 114% and 140% at 22°C and 50°C, respectively, leading also to a reduced range of variation between lines, especially at the highest temperature. However, whatever pretreatment conditions, the line effect stayed highly significant, even when it was greatly reduced. With a solution of 0.2 N NaOH at 22°C, nearly 80% and 70% of cell walls were solubilized in more and less degradable lines, respectively. Such pretreatment conditions limit both energy costs and amounts of effluents to be recycled. Breeding maize lines and hybrids for higher degradability of their cell walls is thus a relevant goal for both animal feeding and environmentally friendly production of bioenergy.

Keywords: maize, bioenergy, pretreatment, alkali, lignin, cell wall degradability

Introduction

Depleted supplies of fossil fuel and environmental damages consecutive of greenhouse gas emissions have lent new urgency to the search for ecological and economic substitutes for transportation and heating. Ethanol currently produced from feed-based substrates (grains, sugars, and molasses) is not a long term energy resource due to the increasing needs for human and animal feeding, but also due to the simultaneous increasing energy demands in developing countries. Energy recovery of crop by-products such as small grain cereal and maize straws is thus a prime competitive and sustainable strategy, after aerobic or anaerobic fermentations with production of bioethanol and biogas, respectively. However, although plant stover contains almost the same amount of gross energy as do grains per unit of dry matter, the stover energy value is significantly lower in both animal digestive tracts and industrial fermenters. The biological conversion of cell wall carbohydrates into fermentable sugars is hindered by the embedding and/or cross-linkages of carbohydrates with lignins or p-hydroxycinnamic acids in plant secondary cell walls. As a consequence, for biofuel production, industrial amenities will most often use a pretreatment of plant biomass. That said, investigations done in the context of maize breeding for dairy cow feeding have shown large genetic variation for cell wall degradability and therefore energy recovery from silage by animals. *In vivo* cell wall digestibility in early and medium early hybrids indeed ranges between 36 and 60% (Barrière et al, 2004a). Correlatively, a similar range from simple to double has been shown for in vitro cell wall enzymatic degradability which varies by a factor of two between 25 and 50%. (Méchin et al, 2000; Barrière et al, 2009).

The purpose of this investigation was then to test whether the differences in cell wall degradability between genotypes still existed after different pretreatments, and accordingly if it was of interest to breed and choose varieties of better cell wall degradability for valorization of crop residues into biofuels, as it was undoubtedly shown for dairy cattle feeding.

Materials and Methods

Plant cropping and sample preparation

Eight early and medium-early lines (Table 1) with increasing cell wall degradability ranging between 25 and 50% were chosen based on previous experiments (Barrière et al, 2009, and INRA lusignan unpublished data). Plants were cropped at INRA Lusignan (Vienne, France) in 2010 using a block design with 3 replicates. Each experimental plot was a two row plot of 5.2 m long with 37 plants per row. Row spacing was 0.75 m, and the density was adjusted to 85,000 plants ha^{-1}. Irrigation was applied during the summer to prevent water stress. When average dry-matter (DM) content of whole plant reached 32 - 35% (silage maturity stage) in the control line F2, ears were

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Table 1 - Cell wall enzymatic solubility (IVNDFD) of the eight investigated lines [IVNDFD is in vitro NDF (Neutral Detergent Fiber) digestibility, Min13 is for Minnesota13 group, RYD is for Reid Yellow Dent group, BSSS is Iowa Stiff Stalk Synthetic group, F line was significant at P < 0.001).

Line	Genealogy	Genetic group relationships	IVNDFD
F4	Etoile de Normandie variety	Northern flint (90%)	49.4
F7084	W117 x F4	Northern flint (65%), RYD (30%), Min13 (15%)	43.9
F324	(F282 x F283) x F286	European flint (75%), Northern flint (25%)	38.8
F7019	[(A632 x B59)-97-2] x F113	Min13 (65%), BSSS (25%)	38.5
EA1301	Razza nano landrace	European flint (60%), Tropical (30%)	36.2
F2	Lacaune landrace	European flint (100%)	34.1
F874	Corn borer tolerance synthetic	European/Caribbean flint (65%), RYD (30%)	25.3
F7033	(Lorena x F252) x F252	BSSS (30%), Lancaster/Min13 (60%),	24.9
Mean			36.4
F line			216.7

removed by hand from all plants the day before harvest in all plots. Plots were then machine harvested with a forage chopper. A representative sample of 1 kg chopped material per plot was collected for DM estimates and biochemical analysis. Samples were dried in a ventilated oven (65°C), and dry samples were ground with a hammer mill to pass through a 1-mm screen for further analyses.

Cell wall analyses and treatments

Because the objective of the work was to investigate treatment effect on cell wall degradability, only the first field replicate was used for each genotype. Considered pretreatments comprised two alkaline concentrations (NaOH 0.02 and 0.2 N), two incubation temperatures (22°C and 50°C), and two periods of incubation (2 and 24 h), with six investigated conditions (2h 22°C, 24h 22°C, 24h 50°C, for both 0.02 and 0.2 N NaOH concentrations). Plant samples (500 mg dry matter) were placed in sealed Ankom filter bags. Pretreatments were conducted in screw cap 50 ml tubes, with 25 ml of alkaline solution, placed in stirring incubators (one sample per tube). Three replicates were done for each experimental condition.

After incubation, bags were washed thrice in tap water. *In vitro* dry-matter enzymatic degradability (IVDMD) was estimated according to Aufrère and Michalet-Doreau (1983). In brief, plant samples were incubated in an Ankom DaisyII incubator during 24 h at 40 °C, with a buffered solution of cellulase (Onosuka R-10, 1 g l^{-1}) and amylo-glucosidase (from Aspergillus niger, Sigma, 1.5 ml l^{-1}). Cell wall content was considered as Neutral Detergent Fiber (NDF), according to Goering and Van Soest (1970). Cell wall digestibility was investigated as in vitro NDF digestibility (IVNDFD) and computed according to Struik (1983) and Dolstra and Medema (1990), assuming that the non-NDF part of plant material was completely digestible [IVNDFD = 100 x (IVDMD - (100 - NDF)) / NDF]. In addition to pretreated samples, control IVNDFD was estimated without any pretreatment and also after dipping in distilled water.

Variance analyses

Variances analyses and mean estimates were investigated considering fixed effect models, including lines, pretreatment conditions, interactions between conditions, and replicates. The «aov» and «effects» procedures of the R software were used for variance analyses and mean estimates, respectively. Analyses were done separately for the different conditions i) NaOH concentrations and temperature, ii) NaOH concentrations and treatment duration, iii) control without any pretreatment, or control after water rinsing.

Results

Line genetic variation for cell wall enzymatic solubility

In the considered set of eight lines (harvested without ears), IVDMD ranged between 53.0 and 69.7%, while IVNDFD ranged between 24.9 and 49.4% (Table 1). The highest cell wall degradability was observed in the F4 line and one of its progeny F7084. The F4 line was bred at INRA Versailles in the 1950' in the early variety Etoile de Normandie, used before the Second World War for grain but also silage production. The lowest values were shown in two lines, one bred in a progeny of a cross between early and late germplasms, and another one bred nearly 25 years ago for corn borer resistance in a synthetic of 16 flint and dent lines. The four other lines had intermediate and rather close IVNDFD values. The observed IVNDFD range of variation is representative of the maximum differences known for this trait in early and medium early lines. Whatever the temperature and duration conditions, water-only pretreatments had no significant effect on cell wall degradability (data not shown).

Table 2 - Variance analysis for cell wall enzymatic solubility (IVNDFD) at two NaOH concentrations and two temperature pretreatments during 24 hours in the eight investigated lines.

IVNDFD	Mean-Square	Fisher
NaOH concentration	1960.0	698.7 ***
Temperature pretreatment	126.8	45.2 ***
Line	330.8	117.9 ***
NaOH x Temperature	306.8	109.4 ***
Line x NaOH	74.7	26.6 **
Line x Temperature	5.4	2.0 ns
Residual	2.7	

F test were significant at P < 0.001 (***),P < 0.01 (**), or non significant (ns).

Table 3 - Line mean and range (maximum - minimum) of degradability values (%) at all investigated conditions, and corresponding F values (significant at P < 0.001).

	Control	IVNDFD (pretreatment 24 h)				IVNDFD (pretreatment 2 h)	
		0.02 N 22°C	0.02 N 50°C	0.2 N 22°C	0.2 N 50°C	0.02 N 22°C	0.2 N 22°C
Mean 8 lines	36.4	50.6	48.3	77.8	87.2	46.2	71.6
Range 8 lines	24.4	24.0	24.3	12.7	8.8	24.8	14.7
F test 8 lines	216.7	266.6	219.1	23.6	10.6	188.1	7.8

Effect of NaOH concentrations and pretreatment temperatures

The effect of NaOH concentration on line IVNDFD was preponderant over the effect of temperature (Table 2), increasing average degradability values by 33 and 6 percentage points, respectively (Table 3, Figure 1). With the most severe conditions nearly 90% and 80% of cell wall components were solubilized in lines of high and low IVNDFD, respectively. Interactions between temperature of pretreatments and NaOH concentrations were high. With the 0.02 N NaOH concentration, lines thus has similar increases in IVNDFD values at the two temperatures, while with the 0.2 N NaOH concentration, all lines got higher IVNDFD values at 50°C than at 22°C. Without alkaline treatment, the IVNDFD range of variation between the eight lines was equal to 24 percent points, and this range remained the same after the NaOH 0.02 N pretreatment. However, ranges between lines were strongly reduced after the 0.2 N NaOH pretreatment, reaching only 50 and 35% of the control values when applied at 22 and 50°C, respectively. Interactions between lines and NaOH concentrations were of weaker importance, but significant, with a greater efficiency of lower NaOH concentration in lines with higher IVNDFD than in lines with lower IVNDFD. Interactions between lines and temperature were non significant. Whatever the conditions, line effects stayed highly significant (P < 0.001), but with decreasing F values when treatment severity increased, especially with 0.2 N concentrations (Table 3).

Effect of pretreatment duration at 22°C

At 22°C, the effect of pretreatment duration was highly significant, but lower than the effect of NaOH concentration (Table 4). The interaction between NaOH concentration and pretreatment duration was significant, with greater IVNDFD when both duration and NaOH concentration were higher. Interactions between lines and NaOH concentration were significant, but of weaker importance, with a little greater susceptibility to low NaOH concentration in lines with higher IVNDFD in control conditions. No interaction was shown between lines and pretreatment durations. Moreover, for both NaOH concentrations, about 90% of average maximum possible degradation was obtained after 2 h of pretreatment, illustrating the great susceptibility of maize tissues to alkaline attacks.

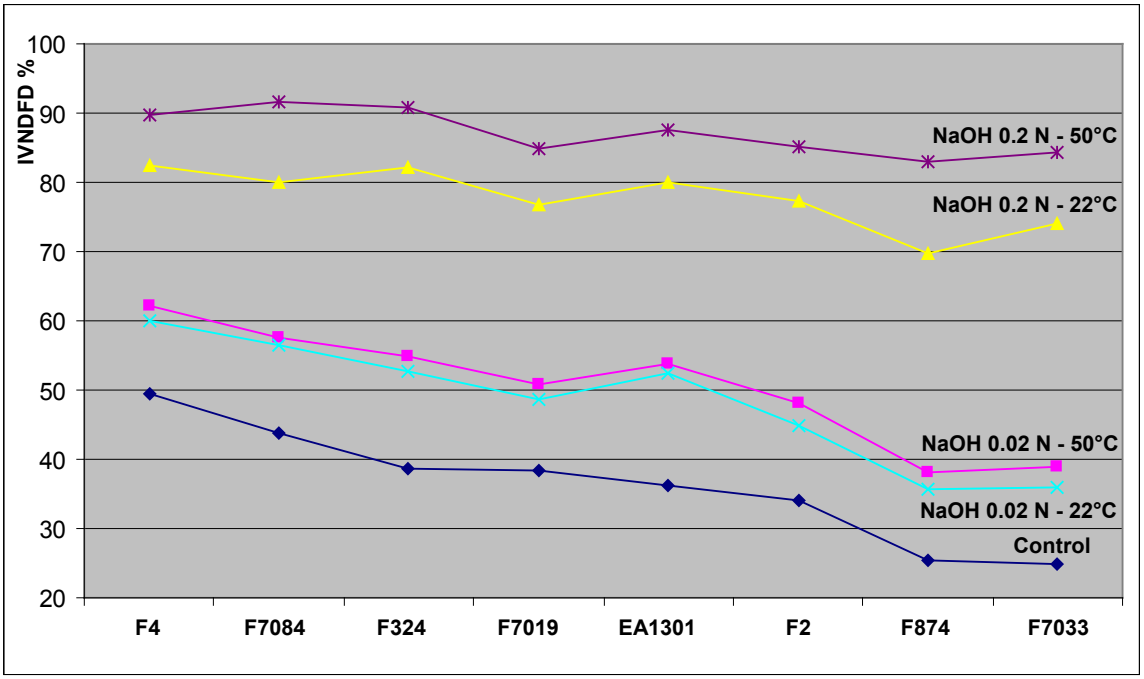

Figure 1 - Cell wall enzymatic degradability (IVNDFD) values in the eight lines according to alkali concentrations and to temperature of pretreatments during 24 h.

Discussion

Table 4 - Variance analysis for cell wall enzymatic solubility (IVNDFD) at two pretreatment durations and two NaOH concentrations at 22°C in the eight investigated line.

IVNDFD	Mean-Square	Fisher
NaOH concentration	1853.4	183.6 ***
Pretreatment duration	1172.1	116.1 ***
Line	382.3	37.9 ***
NaOH x Duration	139.2	13.8 **
Line x NaOH	48.4	4.8 **
Line x Duration	4.0	0.4 ns
Residual	10.1	

F test were significant at $P < 0.001$ (***),$P < 0.01$ (**), or non significant (ns).

Alkaline pretreatments of lignocellulosic materials were shown to cause cell wall structure swelling, with an increase in internal surface area. Another consequence is a decrease in the degree of cell wall component polymerization, with breakage of structural linkages between lignin and carbohydrates, and disruption of the lignin structure. Alkaline pretreatment also reduces cellulose crystallinity, all these modifications making cellulose and hemicellulose more available for the enzymatic degradation (Canilha et al, 2012; Behera et al, 2014).

Several experiments have thus proven the efficiency of alkali pretreatments on further solubilization and sugar releases from grass cell walls. Total sugar release from sorghum straw was 4.3 and 5.6 fold higher after pretreatment in 0.025 N NaOH at 60°C and 121°C, respectively, compared to samples only pretreated at 60°C in absence of alkali. Addition of glucosidase and xylanase to final saccharification mixtures enhanced saccharification efficiency of pretreated samples up to 95% (McIntosh and Vancov, 2010). Similarly, dilute NaOH solution applied to sugarcane bagasse at 180°C allowed, after enzymatic digestion, a total sugar recovery equal to 77.3% of sugar present in untreated bagasse (Yu et al, 2013), taking into consideration that sugarcane bagasse is a lignified and recalcitrant substrate.

The effect of NaOH pretreatment was also investigated on the methane production of ensiled sorghum plants and wheat straws. With a pretreatment done at 40°C with 2.5 N NaOH, the methane production of sorghum forage was enhanced by up to 32%, while the increase was equal to 43% for pretreated wheat straws in similar conditions. When temperature of treatment was raised to 100°C, the increase in methane production reached 67% in wheat straw, but no improvement was observed in sorghum at this higher temperature (Sambusiti et al, 2013). The nearly 60% higher lignin content in wheat straws, in comparison to sorghum silage with more immature tissues, likely explained the greater efficiency in wheat straws of more severe conditions which are then requisite to achieve a more complete cell wall disorganization.

With maize tissues, and with investigated temperature and alkaline pretreatment conditions, the effect of NaOH concentration was much higher (nearly 15 times) than temperature effect. Line effect was also higher than temperature effect, and line interactions with both NaOH concentration and temperature were low. In contrast, the NaOH concentration x temperature interaction was high, of the same order of magnitude than the line effect. The increase of tempera-

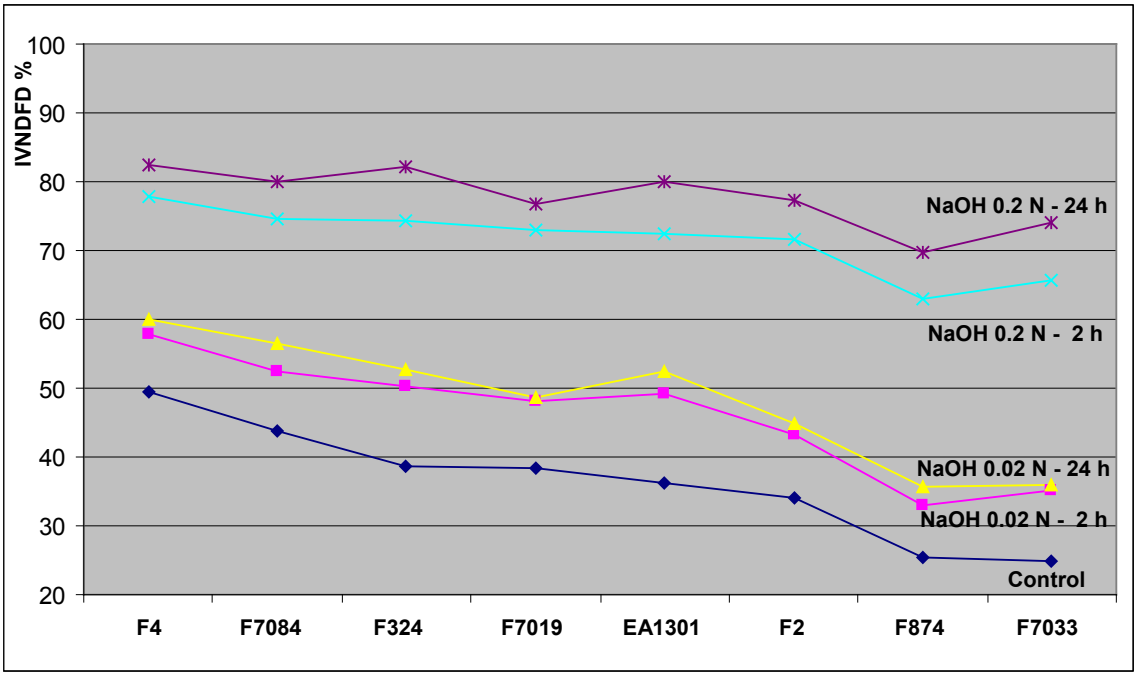

Figure 2 - Cell wall enzymatic degradability (IVNDFD) values in the eight lines according to alkali concentrations and durations of pretreatments at 22°C.

ture from 22 to 50°C with the 0.02 N concentration of NaOH did not improve cell wall degradability whatever the line, while with the 0.2 N concentration of NaOH, a significant increase in degradation was observed at the highest temperature. The genetic variation for cell wall degradation was significant whatever the NaOH concentration and temperature conditions, and was even smaller in more severe conditions. Despite the fact that pretreatment conditions were less severe than those used in published investigations on sorghum, sugarcane, or cereal straws, 80% of the maize cell wall was solubilized in half of the considered lines. Above all, a strong increase in degradability was shown after the 0.2 N alkaline pretreatment at ambient temperature, especially in more genetically degradable lines, which was not observed to a similar extent with less degradable lines. Moreover, in order to reach a given value of cell wall solubilization, stronger conditions were shown to be necessary for lines with lower cell wall degradability in the absence of pretreatment. Corroborating these facts, the enzymatic saccharification of brown-midrib *bmr6* and *bmr18* sorghum after lime pretreatment was shown to be higher than that of normal counterparts, thereby reducing pretreatment costs and effluents (Maehara et al, 2011).

Two limits of these investigations should be considered before applications for maize and grass biofuel variety breeding. The pretreatment efficiency was assessed with the use of enzymatic solubility tests, as it is usually done for forage quality assay. Biomass conversion to fermentable sugars has indeed similarities with forage *in vivo* or *in vivo* digestibility, however these two traits are not synonymous and likely do not fully correspond to similar genetic mechanisms. Moreover, the current results, which were obtained with a limited set of maize lines, have obviously to be validated at larger scales, including especially hybrid plants. However, breeding for a higher degradability of the cell walls is very likely a relevant goal for valorization of crop residues into biofuels (as it was undoubtedly shown for plant green parts in dairy cattle feeding). Such a breeding effort, using well known tools, will very likely allow reducing amounts of energy used and effluents to be recycled during biofuel or biogas productions, and consequently improving the economic and ecological efficiency of this new industrial sector. Finally, this demarche strengthens the possibility of breeding maize hybrids with improved cell wall traits with interest both as silage maize and also for biofuel production from plant straw after grain harvest.

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