Looking at Cell Wall Components with our Customers in Mind

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Abstract

Fiber digestibility of alfalfa for animal nutrition is a complex system encapsulating animal, plant, and microbe biological traits. Understanding all components within the system is key to predicting forage quality. We investigated the relationship between alfalfa cell wall components and *invitro* neutral detergent fiber digestibility (IVNDFD) speed (16-hr) and potential (96-hr) of by cattle ruminant microbes. A composite alfalfa (Medicago sativa L.) population from seven commercial cultivars underwent two cycles of bidirectional selection for plants with low or high stem 16-hr IVNDFD and low or high stem 96-hr IVNDFD. The resulting selected populations were then evaluated by near inferred spectrometry for structural cell wall components and thier relationship with IVNDFD. Hemi-cellulose and cellulose components were found to have a greater negative correlation (-0.85 & -0.86) on the speed of digestion (16-hr IVNDFD) than lignin (-0.70). Whereas, for the overall potential of stem digestibility, lignin (-0.89) had the greatest negative correlation. The relationship between cellulose and lignin with IVNDFD was futher supported with the use of a path model. Lignin and 96-hr IVNDFD had the strongest broad sense heritability across the populations (0.74 & 0.70 respectively). Pectin components correlated positively with speed of digestion (0.41) but had limited correlation on the overall digestibility potential. As IVNDFD increased with each breeding cycle, it remained stable across environments along with concentrations of total cell wall components, lignin, hemi-cellulose, and pectin. However, the cellulose concentrations were not stable across environments. Cell wall components such as hemi-cellulose and lignin could be used as selection traits for increased IVNDFD breeding and may be a way to link invitro digestibility to plant trait genes for genomic selection.

Introduction

Lignification of the plant cell wall is considered the main impediment to forage digestibility (Jung and Allen 1995; Jung and Deetz 1993). Lignification occurs during the formation of secondary cell wall formation. Secondary cell walls use lignin for cell rigidity and are found in cell types such as vascular tissue to resist water pressure. Within alfalfa, stem lignin cell wall concentrations have been reported to be negatively correlated with 96-hr IVNDFD but not 16-hr IVNDFD(Jung and Lamb 2006; Jung and Lamb 2003). However, this appears to be population specific, as other studies found no consistent negative relationship between digestibility of alfalfa and lignin (Jung and Deetz 1993; Lamb et al. 2014). These differences in observations may be related to expressing lignin as a proportion of dry matter rather than of the cell wall (Jung and Deetz 1993). Quantifying lignin is a part of the cell wall fraction it is more appropriate to evaluate its impact on cell wall digestibility compared to dry matter. Because the relationship between lignin and dry matter digestibility is not simple nor constant when used as a breeding target to improve digestibility (Vogel et al. 1981).

The fiber digestibility of alfalfa has been increased through both conventional breeding (Passot et al. 2018) and genetic engineering (Chen and Dixon 2007). Conventional breeding has been successful in increasing invitro dry mater digestibility (IVDMD) and decreasing NDF and ADL concentrations of alfalfa herbage (Coors et al. 1986). The lack of a correlation of IVDMD with ADL concentration suggests that cell wall digestibility of alfalfa had not been altered even though there was a change in forage quality (Sheaffer et al. 2000). Furthermore, while selecting for IVDMD can be effective in improving overall forage quality, it is unlikely to be optimal for improving cell wall digestion, because most non-cell wall comments are 100% digestible in the IVDMD assay. This factor results in the IVDMD selection being for low cell wall concentration (Jung et al. 2012). Therefore, focusing on stem cell wall digestibility, which has known genetic variation (Buxton et al. 1987), would improve overall digestibility of alfalfa. (Jung and Lamb 2006) were successful in selecting for IVNDFD of stems, which measures the digestibility of fiber without accounting for non-cell wall components. Breeding for genetically modified cell wall characteristics has occurred in the form of reduced lignin genotypes. Transgenic genetically modified (GM) reduced lignin cultivars were obtained by the downregulation of the gene encoding caffeoyl coenzyme A-3-O-methyltransferase (CCoAOMT), a component of the lignin biosynthetic pathway (Guo et al. 2001), and marketed under the tradename HarvXtra (Barros et al. 2019). Reduced lignin cultivars contained 8.4% lower in acid detergent lignin (ADL) and 5.3 to 7.7% higher in neutral detergent fiber digestibility (NDFD) than the non-reduced lignin reference cultivars

(Arnold et al. 2019), specifically in the stem portion of the plant (Grev et al. 2020). However, only ADL and sequential fibers (NDF and ADF) were used to estimate cell wall carbohydrates (Arnold et al. 2019; Grev et al. 2020). The digestibility of fiber (i.e. sequential fibers) does not equate to cell wall components.

Sequential fibers are an estimation based on compound breakdown resulting in a gross estimation of cell wall components in a detergent solution. NDF accounts for nutritively available and soluble constituents within the cell, whereas ADF is the portion of the cell that is estimated to undigestible hemi-cellulose, bound cell wall proteins and lignin (Goering 1970). Directly measuring structural carbohydrates that make up cell walls allows for an accurate accounting of cell wall structure and identifies a plant-controlled trait that can be utilized as a breeding target for cell wall digestibility. Therefore, in this study we quantified cell wall carbohydrate in alfalfa populations selected for both high and low IVNDFD. We were able to determine the causal relationships between cell carbohydrates and IVNDFD for improved plant breeding targets.

Methods

The parental population from six commercial alfalfa varieties (5312, Rushmore, Magnagraze, Wintergreen, Windstar, and WL 325HQ) adapted to the Minnesota environment. This population was established at the University of Minnesota Sand Plains Research Farm, Becker, MN as described previously (Jung and Lamb 2006) Plants with low or high rates of fiber digestion (16-hr IVNDFD) and plants with low or high extent of fiber digestion (96-hr IVNDFD) were selected, removed from the field, and grown in the greenhouse for intercrossing between time digestibility timepoint (16 vs 96hr) and trait value (high vs low). Evaluations of nine populations, parental population C0, four C1 populations, and four C2 populations were conducted in 2009-2011 at the Sand Plains Research Farm, Becker, MN (45.37° N, 93.87° W and Hubbard loamy sand), and University of Minnesota Experimental Research Station, St. Paul, MN. The study layout was a randomized complete block design (RCBD) with four replicates. Plots were 1.2 x 1.2 m with plants seeded at 7.6 cm spacings resulting in 16 plants per 1.2 m^{-2}.

Plots were sub-sampled to collect herbage at early bud, flower (25%-30%), and green pod maturity stages in 2010 and 2011. Agronomic traits were collected and recorded. All plants were cut at a 5-cm stubble height at each harvest, dried at 60°C. Plot stems were hand-separated from the leaf material. Stems were then ground to pass a 1-mm screen in a cyclone-type mill and analyzed by NIRS (Foss Model 6500; Foss North America Inc., Eden Prairie, MN) for 16-hr and 96-hr IVNDFD and cell wall traits: neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), Klason lignin (KL), uronic acids (UA), rhamnose (RHA), fucose (FUC), arabinose (ARA), xylose (XYL), mannose (MAN), galactose (GAL), and glucose (GLC). The 138 alfalfa calibration stem samples were assayed for NDF, ADF, and ADL in sequential order (Van Soest et al. 1991) using the Ankom filter bag (Ankom Technology Corp., Fairport, NY) method and sulfuric acid for ADL determination. Cell wall components were determined by the Uppsala dietary fiber method. Uronic acid polysaccharide components were measured color-metrically (Ahmed and Labavitch 1978). The IVNDFD was determined as described previously (Jung and Lamb 2003). All assays were done in duplicate. The NIRS prediction equations were created using the software program Calibrate (NIRS 3 version 4.0, Infrasoft International) with the modified partial least squares regression option.

Pearson correlation was used to estimate the relationship between the digestibility and cell wall components using JMP Pro 15.0.0 (2019 SAS Institute Inc.). Analysis of means was done using Tukey HSD with an α=0.05. The path model was conducted using R v.3.3.2 statistical package *lavaan* and plotted with *semplot.* The initial path model contained 8 measured elements, and 14 parameters. Chi-square and five fit indices were applied to the model to compare model fit. Modifications were made on the model based on best fit measures by adjusting pathways stepwise with modification indices for a final iteration of the model that indicated best fit. Cross populations in cycle 2 of selection (HxH & LxL) were modeled (n= 157 and 152, respectively) and compared to bulked populations across cycles of selection (n=1795). An ANOVA was conducted between final models to determine if the models were significantly different.

Results and Discussion

Breeding populations selected for either high or low digestibility increased or decreased in digestibility for both IVNDFD at 16hr and 96hr based on selection type, however only 96hr was significant (Table 1). This results suggests that traits that impact overall digestibility of the plants have more infulunce genetically than traits that allow for rapid digestion. These traits include lignin and hemicelluosle. The reduction of lignin using transgenetics in alfalfa has been found to increase digestibility over time and extend the time in which high quality alfalfa can be harvested (Arnold et al. 2019; Grev et al. 2020).

Table 1. Average stem in vitro neutral detergent fiber digestibility (IVNDFD), detergent fiber components, and cell wall traits of alfalfa populations across all years, harvests, and maturities. Tukey HSD α=0.05

Digestibility Traits	C0		C1 H x H		C2 H x H		C1 L x L		C2 L x L	
16-hr IVNDFD (g kg^{-1})	189.52	± 20.8	193.58	± 20.0	198.01	± 21.0$^{\mp}$	186.09	± 20.9	182.46	± 19.5 $^{\mp}$
96-hr IVNDFD (g kg^{-1})	432.06	± 36.8	439.62	± 34.2	451.81	± 30.7***	414.45	± 38.1	400.58	± 37.5**
NDF (g kg^{-1} DM)	601.25	± 45.8	592.69	± 43.7	582.27	± 44.5 $^{\mp}$	610.09	± 47.1	617.83	± 43.8
ADF (g kg^{-1} DM)	456.69	± 42.1	449.86	± 40.2	441.92	± 41.2	461.40	± 43.0	468.72	± 40.0 $^{\mp}$
ADL (g kg^{-1} DM)	166.46	± 11.6	162.95	± 11.0	159.84	± 10.6*	170.46	± 12.0	174.72	± 11.4**
CW (g kg^{-1} DM)	646.73	± 83.5	460.80	± 35.3	453.22	± 62.7*	471.94	± 168.9	475.97	± 36.6*
KL (g kg^{-1} CW)	195.33	± 21.7	192.4	± 20.3	185.62	± 19.4***	203.52	± 22.9	207.77	± 24.0*
Cellulose (g kg-1 CW)	417.61	± 41.1	413.85	± 39.2	409.85	± 41.3	417.37	± 42.2	421.09	± 38.1
Hemicellulose (g kg^{-1} CW)	161.52	± 12.2	161.47	± 11.1	160.08	± 11.7**	164.62	± 12.3	165.22	± 11.7
Pectin (g kg^{-1} CW)	218.04	± 11.8	198.15	± 11.1	202.89	± 10.5	190.19	± 11.7	186.62	± 10.6 $^{\mp}$

* Significant level of probability at 0.05, ** 0.01, *** 0.001; $^{\mp}$ significance at α=0.10 level of probability

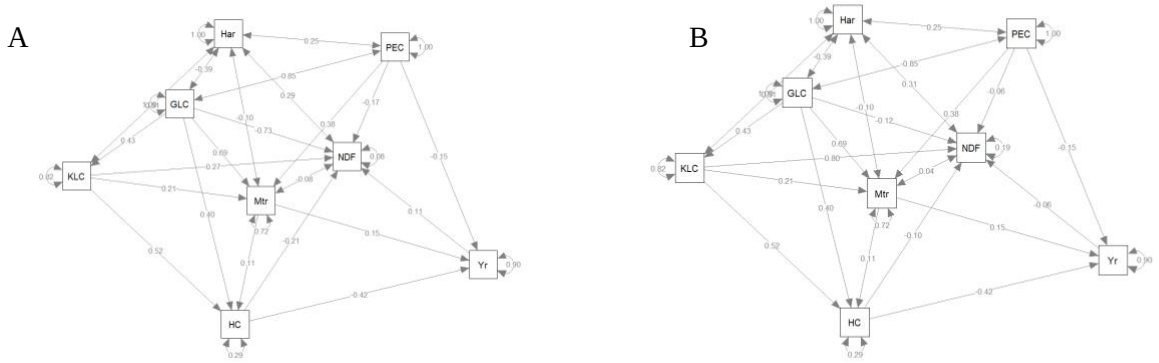

Figure 1. Path analysis showing trait relationships. A. 16hr IVNDFD B. 96hr IVNDFD. Both models contain contained 8 elements and 22 interactions and have a model fit of chi-square: 8.221, $p = 0.22$, df=6; Comparative Fit Index = 1.00; Tucker-Lewis Index = 0.999; Standard Root Mean Square Residual = 0.007; Root Mean Square Error Approximation: 0.014.

All relationship values for both IVNDFD-16hr and 96-hr models irrespective of the population (HxH cycle 2, LxL cycle 2 or bulked) were the same. Therefore, the bulked population was used to describe the relationship between the 8 elements. The bulked population models for IVNDFD-16hr and IVNDFD-96hr were significantly different in their trait relationships ($p > 0.0001$), indicating that the traits that impact rapid digestibility are significantly different from those that impact total digestibility. Klausen lignin had the largest causal relationship with IVNDF-96hr (-0.799) followed by CEL (-0.125), HC (-0.099) and PEC (-0.060). Similar to IVNDFD-16hr, both KL and CEL impacted HC (0.52 and 0.40, respectively) with no direct relationship between HC or KL and PEC. Year negatively impacted IVNDFD (-0.055), while management traits such as maturity and harvest were positively correlated (0.036 and 0.308). Relationships between traits not directly related to IVNDFD were the same in both 16hr and 96hr digestibility models. Forage voluntary food consumption intake (V.F.C.) highly correlated with cellulose digestibility of both dicots and grasses and not lignin concentration (Allinson and Osbourn 1970). The linkage between lignin and cellulose is that as plants mature lignin encapsulated cellulose inhibiting rumen digestion (Jung and Engels 2002; Weimer 1992). This linkage is supported by our path analysis where CEL had a positive causal relationship with KL (z-score = 0.43, p>0.0001) and a negative causal relationship with digestibility (Figure 1.)

Conclusions and/or Implications

The data suggests that reducing cellulose and lignin increases cell wall digestibility of alfalfa for cattle, and can be bred by using IVNDFD as a selection trait. Our study clearly indicates that reducing lignin will increase

the total (96hr) digestibility of alfalfa. However, reducing cellulose, a key component in digestible neutral detergent fiber and a carbohydrate source, to improve rapid cell wall digestibity is counter intuitive. Therefore we suggest that the availiblity of cellulose to rumen microbes should be increased either by the reduction of lignin encapsulation or the reallocation of lignin to key areas of the stem that maintains structural integrity without inhibiting microbe access to the majority of the cell wall cellulose.

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