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Improved Forage Quality in Alfalfa (*Medicago sativa* L.) via Selection for Increased Stem Fiber Digestibility

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Abstract: The low digestibility of fiber in alfalfa (*Medicago sativa* L.) limits dry matter intake and energy availability in ruminant animal production systems. Previously, alfalfa plants were identified for low or high rapid (16 h) and low or high potential (96 h) in vitro neutral detergent fiber digestibility (IVNDFD) of plant stems. Here, two cycles of bidirectional selection for 16 h and 96 h IVNDFD were carried out. The resulting populations were evaluated for total herbage, percentage of stems to total biomass, IVNDFD, neutral detergent fiber (NDF), and acid detergent lignin as a proportion of NDF (ADL/NDF) at three maturity stages. Within these populations, 96 h IVNDFD was highly heritable ($h^2 = 0.71$), while 16 h IVNDFD had lower heritability ($h^2 = 0.46$). Selection for high IVNDFD reduced NDF and ADL/NDF in plant stems at the late flowering and green pod maturity stages and reduced seasonal variability in stem digestibility but did not alter the percentage of stems. Stability analyses across 12 harvest environments found that selection for high IVNDFD had little effect on environmental stability of the trait compared to the unselected population. Thus, selection for stem IVNDFD was a highly effective strategy for developing alfalfa populations with improved nutritional quality without changing the percentage of stems to total biomass.

Keywords: alfalfa; forage quality; lignin; in vitro neutral detergent fiber digestibility; IVNDFD

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1. Introduction

In the United States, alfalfa (*Medicago sativa* L.) is the third largest field crop produced, with an annual estimated value of over USD 10 billion [1]. Primarily used in animal feeds, it is also valued for its diverse environmental services [2] such as carbon sequestration and mitigation of nitrogen leaching from surface water and tile drainage. Alfalfa herbage is an excellent source of nutrients for milk production in dairy cattle and increasing muscle mass in beef cattle [3–5].

Alfalfa herbage is comprised of a protein-rich, highly digestible leaf fraction and a less digestible carbohydrate-rich stem fraction [6]. The stem fraction is higher in indigestible fiber concentration than the leaf fraction, and as the stems mature, the fiber concentration increases within stems while the fiber in leaves remains low and fairly constant [7]. Because stems make up approximately 46 to 60% of the dry matter in alfalfa herbage [8], increasing the digestibility of alfalfa stems would impact the dairy market substantially through increased animal productivity, reduced feed costs, and reduced animal waste [4,9]. Additionally, increasing the available energy of the stem at later maturity stages would also increase dry matter yield [10,11]. Thus, it has been recognized that alfalfa breeding should focus on improving stem digestibility to improve the overall quality of alfalfa forage [12].

In forage quality analysis, plant cell walls (CWs) are typically characterized by the detergent fiber fractions: neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL). Generally, forages that limit feed intake have greater NDF concentrations and more highly lignified CWs [13]. Because alfalfa stems contain more

NDF than alfalfa leaves, morphological changes, such as the leaf-to-stem biomass ratio, usually affect NDF values and digestibility. After alfalfa matures beyond the vegetative stage, there is a rapid increase in NDF concentration and a concurrent rapid decrease in in vitro digestible dry matter [14]. Feeding studies have reported that improving NDF digestibility (NDFD) resulted in significantly greater dry matter intake and milk yield in dairy cows [5]. Therefore, focusing breeding efforts on in vitro neutral detergent fiber digestibility (IVNDFD) may increase alfalfa digestible biomass yield.

Progress in developing alfalfa cultivars with improved stem CW digestibility has been slow due to the quantitative nature of the trait and low heritability [15], even though extensive variability in CW components and IVNDFD occurs in alfalfa germplasm [16]. Further genetic improvement in alfalfa forage digestibility utilizing marker-assisted selection or recombinant DNA technologies will require the identification of biochemical traits and associated genes that impact the rate and/or the extent of CW digestibility.

Fiber digestibility in alfalfa stem CWs has been increased through conventional breeding [17]. Recurrent phenotypic selection was used to develop populations that were evaluated using enzyme-released glucose as a proxy trait for fiber digestibility. The results indicated that digestibility was increased significantly, and the selection method was effective [17]. This trait was correlated with IVNDFD and in vitro total digestibility (IVTD) and had the benefit of not depending on the cyclic and variable rumen composition of different animals. In another breeding program, alfalfa plants were identified with stems that had either low or high rapid (16 h) and low or high potential (96 h) IVNDFD. These plants also differed in stem NDF and ADL as a proportion of NDF (ADL/NDF) [18]. A stability analysis demonstrated that stem NDF and 96 h IVNDFD were more environmentally stable among the alfalfa clones in the field environments than stem 16 h IVNDFD or ADL/NDF, suggesting that stem 96 h IVNDFD, NDF, and ADL as a proportion of NDF would be the traits to use in a field selection program to develop alfalfa with improved nutrient value [10].

In this study, two cycles of bidirectional selection for 16 h and 96 h IVNDFD were carried out on alfalfa stems. This research was designed to evaluate the effectiveness of recurrent selection on stem fiber digestibility for improving the nutritional value of alfalfa. Specifically, the study aimed to measure the genetic gain, heritability, and environmental stability of IVNDFD and determine whether selection altered CW fiber fractions important in forage digestibility. In a subsequent paper, data will be presented on the changes in CW composition associated with the selection for IVNDFD (Heuschele et al., in preparation).

2. Materials and Methods

2.1. Development of Selected Populations

The cycle 0 (C0) parental population, UMN3097 (Table 1), was created by mixing seeds from six commercial alfalfa varieties (5312, Rushmore, Magnagraze, Wintergreen, Windstar, and WL 325HQ) that are adapted to the Minnesota environment [10,18]. As described previously, this population was established at the University of Minnesota Sand Plains Research Farm, Becker, MN [18]. Briefly, in April 1997, approximately 2400 seeds were hand sown to develop a spaced plant nursery with 15 cm within and between the rows. Herbage from individual plants was harvested when approximately 25% of the plants bloomed in spring (June) and early summer (July) in 1998 and 1999. After drying at 60 °C, stems and leaves were hand separated, and stems were ground to pass a 1 mm screen in a cyclone-type mill (SPEX, 8000M, SPEX SamplePrep, Mentuchen, NJ, USA) for further analysis. Each ground stem sample was scanned via near-infrared spectroscopy (NIRS) using a Foss Model 6500 (Foss North America Inc., Eden Prairie, MN, USA) to predict 16 h and 96 h IVNDFD using prediction equations developed previously [18].

Selected plants were divided into four populations from C0: high 16 h and 96 h digestibility (H16 × H96); high 16 h and low 96 h digestibility (H16 × L96); low 16 h and low 96 h digestibility (L16 × L96); and low 16 h and high 96 h digestibility (L16 × H96). The selected plants within each population were removed from the field and grown in the greenhouse for intermating. Plants were intercrossed by hand tripping flowers without

emasculation. A Cycle 1 (C1) population for increased rate and extent of fiber digestion, UMN3355 (Table 1), was developed via random intercrossing of the plants identified with the highest 16 h IVNDFD and highest 96 h IVNDFD (H16 × H96) utilizing a total of 117 parents. Similarly, a divergent population, UMN3358, was developed via random intercrossing of plants with the lowest 16 h IVNDFD and 96 h IVNDFD (L16 × L96) using 33 parents. The number of intercrossing parent lines was determined based on availability and is listed in Table 1. To test gene effects, Population UMN3356 was developed by intermating plants with high 16 h IVNDFD and low 96 h IVNDFD (H16 × L96), and Population UMN3357 was developed by intermating plants with low 16 h IVNDFD and high 96 h IVNDFD (L16 × H96). For each population, equal amounts of seed were bulked from each plant for use in the next selection cycle. Seeds from the four C1 populations were established in a spaced plant nursery in 2001, as described above for the parental population. As with the first cycle, herbage was harvested three times a year over two years (2002 to 2003) from individual plants, was separated into leaf and stem fractions, and the stem fraction was analyzed via NIRS for 16 h and 96 h IVNDFD. Selected plants were removed from the field for intercrossing in the greenhouse as described for C1 to develop Cycle 2 (C2) populations UMN4016 (H16 × H96), UMN4017 (H16 × L96), UMN4018 (L16 × H96), and UMN4019 (L16 × L96) (Table 1).

Table 1. Summary of populations used in the study. H16, high 16 h IVNDFD; H96, high 96 h IVNDFD; L16, low 16 h IVNDFD; L96, low 96 h IVNDFD. C0, unselected population; C1, cycle 1 population; C2, cycle 2 population.

Population Names	Population Combinations	Cycle Number	Number of Plants Intermated
UMN3097	Parental	C0	
UMN3355	H16 × H96	C1	117
UMN3356	H16 × L96	C1	28
UMN3357	L16 × H96	C1	26
UMN3358	L16 × L96	C1	33
UMN4016	H16 × H96	C2	60
UMN4017	H16 × L96	C2	~30
UMN4018	L16 × H96	C2	~30
UMN4019	L16 × L96	C2	~30

2.2. Evaluation of Selected Populations

Experimental plots were established in 2009 at the Sand Plains Research Farm, Becker, MN, and the University of Minnesota Experimental Research Station, St. Paul, MN, with the parental population C0, and C1 and C2 populations. The plots at Becker were irrigated to meet plant moisture needs using the checkbook method [19], while plots in St. Paul were rainfed. The study layout was a randomized complete block design (RCBD) with four replicates. Plots were 1.2 × 1.2 m with plants seeded at 7.6 cm spacings resulting in 16 plants per 1.2 m^2. A border of the alfalfa cultivar Agate was planted around each side of the plots to prevent edge effects. The experiment layout was a rectangle of 36 ranges by four rows in both Becker and St. Paul, Minnesota. Plots were managed with best practices, and pesticides were applied as needed. Fertilizers were used based on the recommended optimal level of P and K for high alfalfa yields [20].

Plots were sub-sampled in 2010 and 2011 to collect herbage at the early bud (EB) stage in which one or two nodes had visible buds with no flowers or seed pods present, at the late flowering (LF) stage when 25–30% of plants had open flowers, and at the green pod (GP) maturity stage in which a plant had more than four nodes with green seed pods. For each plot, the number of stems was counted, and the average number of stems per plant was calculated. All plants were cut at a 5 cm stubble height at each harvest, dried at 60 °C, and stems were hand-separated from the leaf material. Data on total herbage dry weight, leaf dry weight, stem dry weight, and percent stem were collected. Stems were then ground

to pass a 1 mm screen in a cyclone-type mill and analyzed via NIRS for 16 h and 96 h IVNDFD, NDF, and ADL as a proportion of NDF [18].

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. A set of 138 samples, selected via stratified random sampling to provide an equal representation of each germplasm source, year, and harvest of the study, was used to calibrate the NIRS stem equations (Supplemental Table S1). The 138 alfalfa calibration stem samples were assayed for NDF, ADF, and ADL in sequential order [21] using the Ankom filter bag method and sulfuric acid for ADL determination [22]. The ADL was adjusted using NDF following the approach by Jung and Lamb [18]. The IVNDFD was determined as described previously by Jung and Lamb [22]. Rumen fluid was collected approximately 3 h post-feeding from a fistulated, lactating Holstein cow (*Bos taurus* L.). Samples were placed in Ankom filter bags (Ankom Technology Corp., Fairport, NY) and then incubated with a 20:80 (*v/v*) mixture of rumen fluid and McDougall's buffer [23] at 39 °C for 16 h and 96 h in an Ankom Daisy oven. After the incubation, residues within the filter bags were extracted with the neutral detergent solution as described for dietary fibers [24] to calculate IVNDFD. All assays were conducted in duplicate. All filter bag assays contained an assay blank composed of glass fiber to correct for indigestible NDF entering the bags from the rumen fluid inoculum.

2.3. Statistical Analyses

A five-way analysis of variance (ANOVA) was conducted to estimate the interaction relationships between year (Y), location (L), genotype (G), maturity (M), and harvest (H) via function "aov" from the R package "stats" [25]. The R code for the model with all five main effects and all of the interactions was aov (digestibility ~ Y × L × M × H × G, data = digestibility.data). Fisher's protected LSD test was applied to conduct the pairwise analysis via a function of LSD.test [26] from R package "agricolae" with alpha = 0.05.

Pearson correlation was used to estimate the relationship between the digestibility traits. Functions "cor" and "corrplot.mixed" from R package "corrplot" [27] were employed to estimate the correlation coefficients.

The function "slope.test" from the R package "smart" was used to test whether the regression of the genetic gain was significantly different from 0 using the ordinary least squares method [28]. The rate of genetic gain for all CW traits and IVNDFD was estimated using the best linear unbiased predictions (BLUPs) obtained from the linear mixed model as follows:

$$\text{Genetic gain rate (GGR)} = (\text{BLUPs\_C2} - \text{BLUPs\_C0})/\text{BLUPs\_C0} \times 100$$

BLUPs_C2 are values of the variables from C2 and BLUPs_C0 are the BLUP values of the variables from C0.

Broad sense heritability was calculated for CW traits and IVNDFD as follows:

$$h^2 = V_g/V_p = V_g/(V_g + V_e/r)$$

V_g stands for genotypic variance, V_p stands for phenotypic variance, V_e is the variance of the residuals, and r is the number of replicates for each measurement [29]. Broad sense heritability was calculated with data from both years and locations.

Linear regression was used to estimate whether genetic gain was significantly larger than 0. The significance of the slope was tested with function "slope.test" of an R package "smatr" [28].

slope.test(y = digestibility, x = cycle.number, alpha = 0.05, method = "OLS", robust = True, test.value = 0)

Where alpha is the desired confidence level for the 100(1 − alpha)% confidence interval for the common slope; using the ordinary least square method, Robust = True shows that a robust regression was performed with all of the data and treats all of the data as true data

compared with outliers. The "Test.value" = 0 is to test the null hypothesis of whether the slope is statistically equal to 0.

Digestibility variables were classified into three categories: Best: *p*-value of the slope <0.001 and genetic gain rate >0.5., Ok: *p*-value of the slope <0.05 and genetic gain rate <0.5, or No: *p*-value of the slope >0.1.

All figures were generated using the R package "ggplot2" [30] and "ggpubr" [31].

2.4. Environmental Stability Analysis

Stability analysis evaluates the environmental stability of a genotype relative to the overall experimental mean of all genotypes [32]. The environmental index for 16 h and 96 h IVNDFD, detergent fiber components, and CW compositional traits was calculated by subtracting the mean value for each trait over each of the 12 harvest environments (two locations, three harvests per location from 2 years' experiments) from the overall experimental mean. Linear regression analysis was used to compare the performance of an individual population across the 12 harvest environments to the calculated overall environmental index. The stability analysis was conducted with a function of genotype by environment regression "ge_reg" of R package "metan" for multi-environment trial analysis [32]. The slope of regression (b) was used to evaluate population environmental stability for each cycle of selection using the Type II stability definition of Lamb et al. [10]. A stability coefficient b = 1 indicated that the response of the population to different environments was parallel to the average response of all other populations and was stable across environments. If b > 1, the response of the population was greater than the average response to favorable environments, or conversely, the response was less than average in unfavorable environments. If b < 1, the response was greater than the average in unfavorable environments or less than average in response to favorable environments.

3. Results

3.1. Response to Selection for IVNDFD

The main effects of the year (Y), location (L), genotype (G), plant maturity (M), and harvest (H) were highly significant ($p < 0.001$) for stem 16 h IVNDFD (Table 2), indicating that all five factors affect stem digestibility. However, among the 15 two-to-five-way interaction terms for 16 h IVNDFD, only 3 of the 15 interactions, H:G, M:G, and L:M:G, had significant interactions (*p*-values < 0.01). There were no significant interactions between genotype by year or between genotype by location. There were no complex interactions among the four and five factors with genotypes. The most significant interaction was among maturity, harvest, and year ($p = 2.80 \times 10^{-208}$), with the second-largest interaction occurring between maturity and harvest ($p = 2.50 \times 10^{-162}$) (Supplemental Table S2), indicating that maturity and harvest time are the most critical factors for stem digestibility. While the interaction with maturity is expected, the interaction of harvest suggests that the time of sampling is a critical factor for the selection of plants for 16 h IVNDFD. The lack of interaction of genotype with location and year indicates the environmental stability of selected traits and will be addressed more fully later. Across locations, harvests, and maturities, stem 16 h IVNDFD was strongly negatively correlated with NDF (−0.94) (Figure 1).

In contrast to the limited interactions observed with 16 h IVNDFD, significant interactions were found for 96 h IVNDFD (Supplemental Table S2) between germplasm and year ($p = 0.0059$, $p < 0.01$, respectively) and germplasm and location ($p = 0.02$, $p < 0.05$, respectively) as well as between germplasm with year and location ($p = 0.00012$, $p < 0.001$, respectively). Interactions between year and location suggest that the environment plays a significant role in determining the extent of fiber digestibility when digestion time increases from 16 to 96 h. Stem 96 h IVNDFD was negatively correlated with NDF (−0.73), but the correlation strength decreased compared with 16 h IVNDFD from −0.94 to −0.73 for NDF (Figure 1).

Interestingly, the percentage of stems (Per_stem) was the most stable trait with no interactions among any of the five factors except for one significant interaction among location, maturity, and genotype with a *p*-value = 0.047. The observation of low interactions for the percentage of stems indicates that environmental factors did not affect the percentage of stems. Stem dry weight (SDW) was significantly affected by maturity and harvest, and stem dry weight significantly interacted with germplasm (Table 2). There were significant negative correlations of stem dry weight with 16 h IVNDFD (−0.47).

Table 2. Mean squares summary from the analysis of variance (ANOVA) for 16 h IVNDFD (NDFD16), 96 h IVNDFD (NDFD96), acid detergent lignin (ADL) as a proportion of NDF, neutral detergent fiber (NDF), stem dry weight (SDW), and percent stems (Per_stem) among the following five variables: year (Y), location (L), germplasm (G), maturity (M), and harvest (H).

Term	NDFD16 g kg^{-1}	NDFD96 g kg^{-1}	ADL g kg^{-1} NDF	NDF g kg^{-1} DM	SDW g plant^{-1}	Per_stem %
Y	61,131 ***	37,685 ***	3868 ***	79,412 ***	851 *	1471 ***
M	136,873 ***	292,141 ***	20,875 ***	789,443 ***	71,707 ***	21,300 ***
L	11,393 ***	191,234 ***	49,474 ***	12,798 ***	3510 ***	996 ***
H	20,023 ***	39,845 ***	10,591 ***	62,982 ***	106,104 ***	33,054 ***
G	5023 ***	51,724 ***	5257 ***	27,206 ***	2587 ***	201 ***
H:G	173 ***	846 ***	31	919 ***	317 *	24
L:G	89	768 *	100 ***	689 *	464 *	15
M:G	136 **	1609 ***	75 **	823 ***	714 ***	33
Y:G	99	893**	61	599	118	18
L:H:G	66	312	24	320	109	25
L:M:G	137 **	756 **	50	478	914 ***	38 *
M:H:G	70	256	19	372	128	25
Y:H:G	89	274	32	383	55	17
Y:L:G	142 *	1258 ***	128 ***	505	164	15
Y:M:G	38	337	53	270	236	18
L:M:H:G	47	278	24	268	273	22
Y:L:H:G	50	184	20	270	71	18
Y:L:M:G	66	430	37	386	193	34
Y:M:H:G	60	282	17	219	114	24
Y:L:M:H:G	40	120	15	201	110	14

* Significant at the 0.05 level of probability. ** Significant at the 0.01 level of probability. *** Significant at the 0.001 level of probability.

3.2. Genetic Gain by Cycle for IVNDFD and Fiber Digestibility

Stem 16 h and 96 h IVNDFD increased with each cycle of selection in the H16 × H96 populations and decreased in the L16 × L96 populations (Figure 2). A faster gain was seen for 96 h IVNDFD (b = 9.8) compared to 16 h IVNDFD (b = 4.0) from the H16 × H96 population (Figure 2). In contrast, the L16 × L96 populations had negative trait improvement gains, with negative improvement rates (b = −3.7 and −15.0 for 16 h IVNDFD and 96 h IVNDFD, respectively). NDF and ADL as a proportion of NDF increased with selection in the L × L populations and decreased in the H16 × H96 populations compared to the parental population. In the H16 × H96 populations, stem dry weight decreased with selection; in the L16 × L96 populations, stem dry weight increased with selection. Percent stem in the H × H and L × L populations did not change across the selection cycles (Supplemental Table S3).

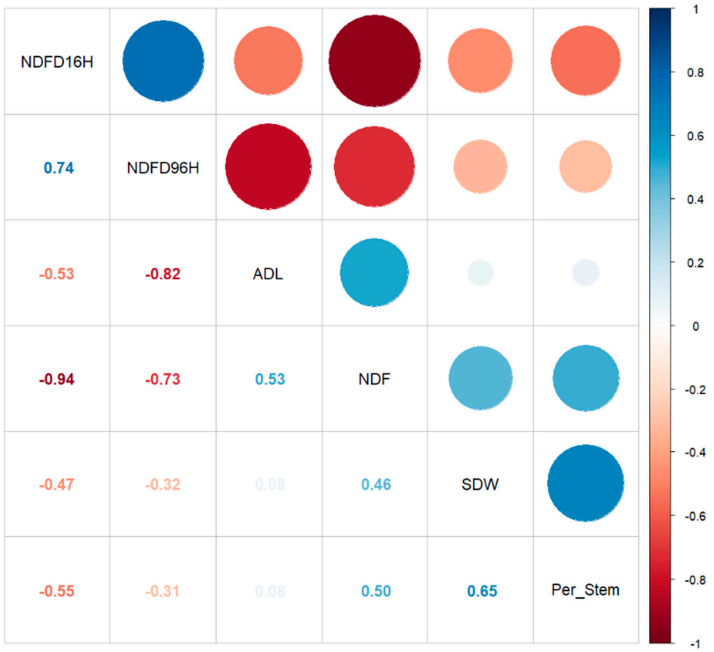

Figure 1. Pearson correlation among the digestibility and CW traits. Numeric values in the bottom lower left section are the correlation coefficients. Names along the diagonal are the variables. Size and color of the circles on the top right section show how strongly the variables are correlated.

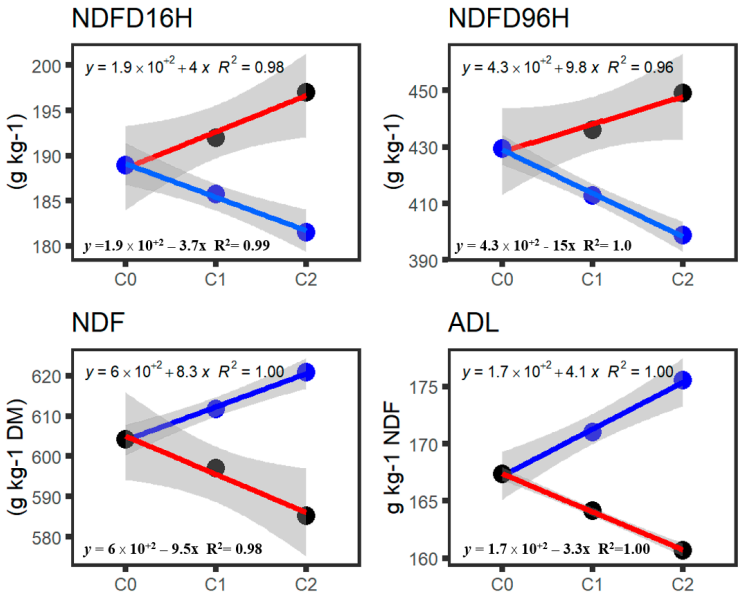

Figure 2. Responses in IVNDFD and CW components over two cycles of divergent selection. Populations selected for high 16 h and high 96 h IVNDFD are in red, and populations selected for low 16 h IVNDFD and low 96 h IVNDFD are in blue. Gray shading around the regression line is the 95% confidence interval around the mean across years, locations, harvests, and maturities. Regression equations and R^2 over the two cycles are shown above or below each line.

3.3. Heritability and Genetic Gain of IVNDFD and Detergent Fiber Components

The values for broad sense mean heritability of fiber digestibility (16 h and 96 h IVNDFD) were significantly greater than the statistical threshold of 0.15 (Table 3). The high heritability of 96 h IVNDFD is likely linked to changes in ADL/NDF, which was highly negatively correlated with 96 h IVNDFD (ADL/NDF −0.82, $p < 0.001$) (Figure 1). ADL/NDF also had the highest heritability (0.75) and high rates of genetic gain (−3.98)

(Table 3). Selection for IVNDFD did not result in significant changes in stem dry weight or the percentage of stems (p-values > 0.05).

Table 3. Summary of heritability, genetic gain (GG), genetic regression slope, and p-values of the regression.

Trait	Heritability	GG	Slope	p-Value
ADL (g kg^{-1} NDF)	0.75	−3.98	−3.42	1.17×10^{-9}
NDFD96H (g kg^{-1})	0.70	4.54	10.13	2.50×10^{-9}
NDF (g kg^{-1} DM)	0.47	−3.15	−9.79	4.60×10^{-6}
NDFD16H (g kg^{-1})	0.46	4.27	4.18	1.84×10^{-5}
SDW_gm (g plant^{-1})	0.15	−16.1	−1.31	8.34×10^{-2}
Per_Stem (%)	0.11	−2.02	−0.01	2.62×10^{-1}

3.4. Effects of Plant Maturity and Harvest on IVNDFD

The stem digestibility of the 16H × 96H populations was measured at three stages, early bud (EB), late flowering (LF), and green pod (GP), over three harvests each year to determine how selection affected digestibility and fiber traits during plant development. The mean 96 h IVNDFD increased with each selection cycle, and the increase was greater at the late flower and green pod stages (Table 4). There was no significant change in 96 h IVNDFD at the early bud stage among the selection cycles. At the late flowering and green pod stages, the second harvests showed the greatest differences among the selection cycles (bold fonts). Overall, the increase in 96 h IVNDFD was greater for C2 than for C1 across the three maturities and harvests (Table 4).

Table 4. Responses in 16 h and 96 h NDFD in the H16 × H96 populations with cycles of selection at the early bud (EB), late flowering (LF), and green pod (GP) maturity stages over three harvests. LSD is Fisher's least significant difference test with different letters within a maturity stage indicating a significant difference; %/cycle is the percent change from C0 to C1 and C1 to C2.

Maturity	Harvest	Cycle	NDFD 16H (g kg^{-1})	LSD 16H	%/cycle 16H	NDFD 96H (g kg^{-1})	LSD 96H	%/Cycle 96H
EB	1	C2	218	a	2.25	485	a	2.03
EB	1	C1	213	a	3.02	476	a	0.25
EB	1	C0	207	a		475	a	
EB	2	C2	210	a	1.39	468	a	1.27
EB	2	C1	207	ab	0.89	462	a	1.55
EB	2	C0	205	b		455	a	
EB	3	C2	192	a	3.44	440	a	3.84
EB	3	C1	186	a	−0.41	424	a	−1.28
EB	3	C0	187	a		430	a	
LF	1	C2	182	a	0.54	442	a	2.47
LF	1	C1	181	a	3.81	431	a	4.11
LF	1	C0	174	b		414	b	
LF	2	C2	188	a	1.50	456	a	3.86
LF	2	C1	186	ab	3.05	439	b	2.86
LF	2	C0	180	b		427	c	
LF	3	C2	197	a	4.27	445	a	5.33
LF	3	C1	189	ab	1.43	422	b	1.21
LF	3	C0	186	b		417	b	
GP	1	C2	178	a	1.66	417	a	3.22
GP	1	C1	175	ab	1.29	404	b	1.42

Table 4. *Cont.*

Maturity	Harvest	Cycle	NDFD 16H (g kg^{-1})	LSD 16H	%/cycle 16H	NDFD 96H (g kg^{-1})	LSD 96H	%/Cycle 96H
GP	1	C0	173	b		398	b	
GP	2	C2	191	a	4.75	450	a	4.08
GP	2	C1	182	b	2.81	433	b	3.65
GP	2	C0	177	b		417	c	
GP	3	C2	199	a	4.11	452	a	5.09
GP	3	C1	191	b	1.91	430	b	2.41
GP	3	C0	188	b		420	b	

3.5. Additive Gene Effects May Control Alfalfa Stem Digestibility

The patterns and trends in 96 h IVNDFD for the four different populations suggest that additive genes may control this trait. In the H × H populations, 96 h IVNDFD increased with each cycle of selection at the three maturity stages, EB, LF, and GP, suggesting the accumulation of positive alleles from C0 to C2 (Figure 3A), and this trend was similar in the first, second, and third harvests (Figure 3B). In contrast, 96 h IVNDFD for the L × L populations decreased with each cycle of selection, suggesting the stacking of negative alleles. There were no unidirectional increases or decreases in the H × L and L × H populations.

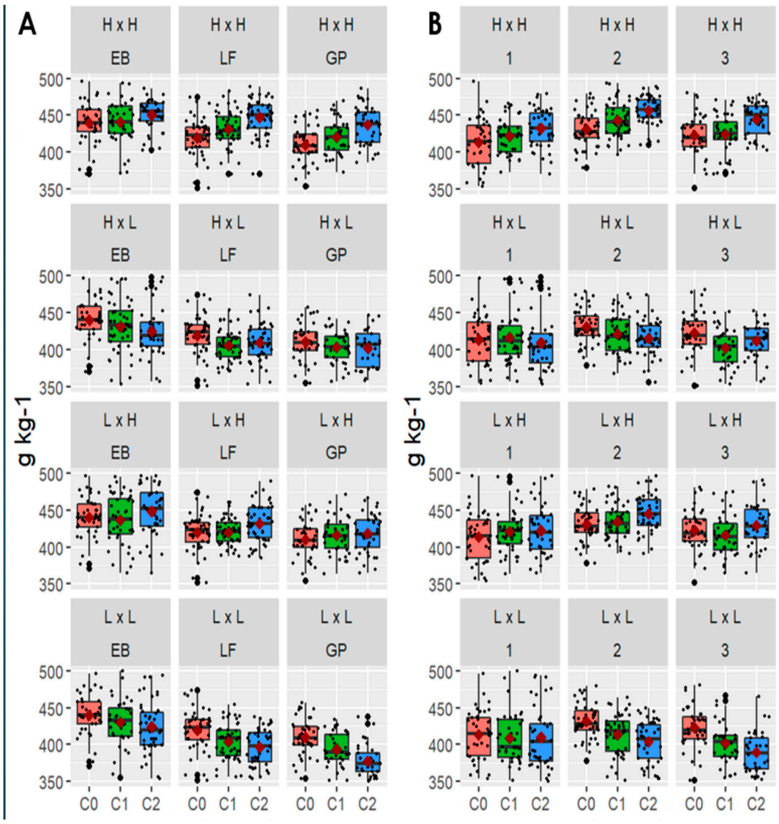

Figure 3. Effect of maturity and harvest on 96 h IVNDFD in selected populations. (**A**) Ninety-six h IVNDFD at early bud (EB), late flower (LF), and green pod (GP) maturity stages across two years, two locations, and three harvests. (**B**) Ninety-six h IVNDFD for combined maturity stages at Harvest 1, 2, and 3 across two years, two locations, and three maturities. The small dots are the individual values, and the large black dots are outliers. The red diamond inside each box is the mean value. C0, unselected population; C1, cycle 1 population; C2, cycle 2 population; H × H, intermating of plants with high 16 h IVDFD and high 96 h IVNDFD; L × L, intermating of plants with low 16 h IVDFD and low 96 h IVNDFD.

3.6. Stability of IVNDFD with Cycles of Selection

Complex genotype × environment interactions for forage quality traits in alfalfa present a challenge to breeders in analyzing germplasm performance. Stem IVNDFD, ADL/NDF, and NDF were shown in this experiment and previous experiments [10] to be impacted by the environment. Environmental stability was measured for each of these traits across the 12 harvest environments for the H16 × H96 and L16 × L96 populations. Stability coefficients for 96 h IVNDFD decreased with selection cycles from 1.08 (C0) to 0.93 (C2) for the H × H populations (Figure 4). In contrast, stability coefficients increased in the L16 × L96 populations with selection cycles from 0.93 to 1.04 and from C0 to C2, respectively. The stability of 16 h IVNDFD followed similar patterns (Supplemental Figure S1). A stability coefficient of b > 1 indicates that the response of the population was better than the average response in favorable environments, while b < 1 suggests that the population performed better than average in unfavorable environments. Therefore, for 96 h IVNDFD, the C2 H16 × H96 population performed better in the less favorable environments, and the C2 L16 × L96 population performed better than average in the favorable environments. Overall, 16 h and 96 h IVNDFD were considered to be environmentally stable in H × H and L × L populations.

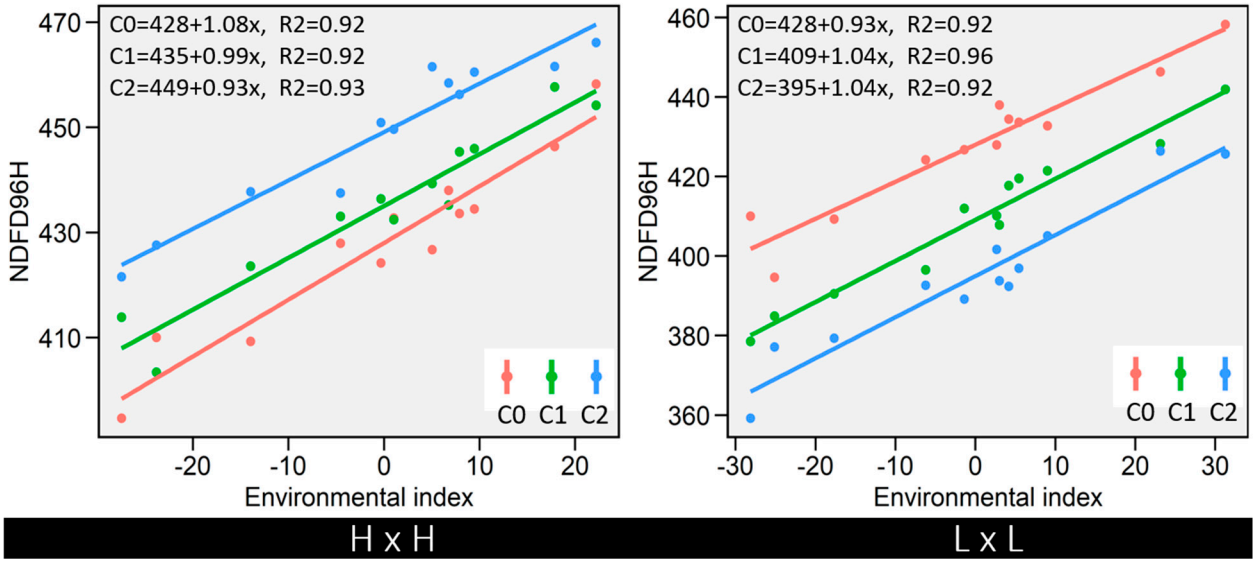

Figure 4. Environmental stability of 96 h IVNDFD with selection cycles across the 12 harvest environments. (**Left**) H × H, intermating of plants with high 16 h IVDFD and high 96 h IVNDFD. (**Right**) L × L, intermating of plants with low 16 h IVDFD and low 96 h IVNDFD.

4. Discussion

Recurrent selection for alfalfa stem IVNDFD was a successful strategy for reducing ADL as a proportion of NDF and NDF in stems without changing the proportion of leaves to stems in total herbage. Increased digestibility and decreased lignin occurred in the later maturity stages, late flowering and green pod, which would increase biomass yields while maintaining forage quality. The selection also reduced seasonal variability in stem digestibility.

Alfalfa leaves are more digestible than stems; therefore, increases in the proportion of leaves to stems result in significant changes in herbage quality [8]. A higher leaf-to-stem ratio is also positively correlated with forage intake [33]. When decreased total herbage lignin concentration was used as a selection trait, overall forage quality increased due to a larger fraction of leaves but the digestibility of the fiber fraction remained unchanged [33,34]. The current study focused on improving stem fiber digestibility to increase forage quality. Using this approach, we found that the percent stem did not change significantly with the

increase in stem IVNDFD over each cycle of selection (Table 3). The regression slope for the percentage of stems was close to 0 ($p = 0.26$) from C0 to C2 (Table 3). The percentage of stems was the most stable trait with the least G × E interaction, which further indicated that the increase in digestibility was due to the improved fiber digestibility of the stems, rather than the proportion of leaves, which changes with plant maturity.

Alfalfa cultivars with improved total herbage digestibility have been developed using traditional breeding methods and genetic modification. The improved total herbage digestibility for conventionally bred Hi-Gest cultivars resulted from an increase in the leaf-to-stem ratio [35,36], whereas the improved forage nutritive value of genetically modified HarvXtra alfalfa was primarily a direct result of changes in ADL and IVNDFD within the stem fraction from the downregulation of caffeoyl coenzyme A-3-O-methyltransferase in the lignin biosynthetic pathway [37,38]. A detailed morphological evaluation of HarvXtra alfalfa in Minnesota reported that HarvXtra and conventional alfalfa cultivars had similar leaf-to-stem ratios that changed similarly with advancing maturity [38]. However, a multistate study found that HarvXtra had a higher leaf-to-stem ratio than the reference alfalfa [39]. The populations developed in the current study appear to be unique, although more research is needed to investigate the basis of increased stem IVNDFD. From observations of CW development and digestion in rumen fluid, Jung and Engles speculated that the rate of alfalfa stem CW digestion could be improved by lowering the syringyl to guaiacyl monolignol ratio in lignin, by reducing the amount of lignification, specifically in the secondary xylem, or by increasing pectin content [40]. Chemical analyses of alfalfa genotypes varying in CW digestibility indicated that the extent of CW digestion could be enhanced by decreasing total CW concentration and selecting for the reduced amount of xylem deposition with maturity [22].

Forage digestibility is measured by the rate and extent of CW breakdown, with rate measured by 16 h IVNDFD and the extent measured by 96 h IVNDFD. When selecting for plants with a high rate and/or extent of stem CW breakdown, the genetic gain and heritability of 16 h IVNDFD was lower compared to the genetic gain and heritability of 96 h IVNDFD. Therefore, breeding progress in increasing the rate of fiber digestibility will be slower than progress in increasing the extent of fiber digestion. The results from this study suggest that more genes may be involved in controlling the rate of digestion than in the extent of digestion. Additive gene control for 96 h IVNDFD was supported by the increase in digestibility in the H16 × H96 populations with each cycle of selection, likely from the accumulation of favorable alleles, while the decrease in digestibility in the L16 × L96 populations was likely from accumulated undesired alleles with each cycle. However, the rates of genetic gain were relatively low, from 4 to 10% in the H16 × H96 populations and −4% to −15% in the L16 × L96 populations. A low rate of genetic gain has been linked to strong genotype by environmental interactions and complex genetic architecture in alfalfa [41,42]. The populations in this study had limited genotype to environment interactions, suggesting that the slow rate of gain was due to either the genetic complexity of the trait or due to the fact that testing locations were not sufficiently diverse to detect interactions.

The heritability of ADL/NDF was high, 0.75, while NDF in the selected populations was moderate, 0.47. These traits are negatively associated with forage digestibility and negatively associated with forage intake [43]. Previously, divergent selection for ADL in total herbage indirectly affected NDF [33,34]. When populations were selected for high ADL, the NDF increased in total herbage and plants had shortened stem internodes compared to the low ADL populations. In the previous study, the improved digestibility occurred due to the dilution of stem NDF by the greater proportion of highly digestible leaves in total herbage. Thus, in this study, the selection for stem IVNDFD improved traits other than leaf digestibility traits.

Most alfalfa is harvested at the early bud stage to maximize forage quality. For ruminant animals, digestibility decreases as plants mature due to the deposition of lignin in vascular cells and the cross-linking of lignin with cellulose microfibrils, as well as the

cross-linking of cellulose and hemicellulose. However, harvesting at the early bud stage sacrifices herbage biomass yield potential, increases stress on the plant, and incurs greater financial costs for harvesting since more harvests are required each season.

Harvest timing can also affect alfalfa forage quality. The first harvest, usually in late May in Minnesota, is typical of high quality due to the high leaf-to-stem ratio, but quality often decreases in the hotter and drier harvests obtained in July and August, particularly for later maturity stages. Selection for high IVNDFD resulted in populations that had increased digestibility and reduced ADL/NDF at the second and third harvests (Supplemental Table S4). Selection for low IVNDFD resulted in populations that had reduced digestibility and increased lignin at all three harvests. Thus, the selection methodology resulted in populations with the desired characteristics at later maturities and across harvests, reducing seasonal variation in forage quality.

Improving forage digestibility may impact forage yield. For example, alfalfa from divergent selections for total herbage and low lignin had lower DM yields than high-lignin lines [34]. This was attributed to the reduced main stem length of the low lignin lines. Additionally, genetically modified HarvXtra, with increased stem digestibility, had a lower biomass yield in a multistate study [39], although no biomass yield differences were found compared to conventional lines in a single-state study [37,38]. Despite a trend in yield differences between populations from selection C0 to C2, the only significant decrease in herbage dry weight (HDW) occurred at the green pod maturity stage for the C2 H16 × H96 population (Supplemental Table S5). The yield decreased because, during the second year, the number of plants per plot decreased later in the growing season—especially in the GP plots. This result indicated that an increase in alfalfa stem digestibility was not achieved at the expense of HDW at the early bud and late flowering maturity stages. Similarly, recurrent phenotypic selection for higher stem enzyme-released glucose concentration (i.e., higher digestibility) in mature alfalfa was shown to improve CW digestibility without affecting alfalfa DM yield [17]. Our study relied on hand sampling for determining HDW; thus, the future testing of herbage yields of improved populations in larger plots with mechanical harvesting is warranted.

The pattern of change in 96 h IVNDFD from the four populations shows apparent additive gene effects (Figure 3). The unidirectional increases in the H × H selection or decreases in the L × L selections are evidence of additive effects, while the lack of a pattern in the L × H and H × L populations indirectly confirms the hypothesis of additive effects. However, epistasis cannot be ruled out if the gene interactions are stable for specific germplasm groups as heterotic groups. In addition, stem digestibility is a complex trait, and genome-wide association studies (GWAS) confirmed the polygenic control of forage quality traits [44]. These studies found that many marker-trait associations differed depending on the environment, although a few were consistent across environments [41,45]. Thus, genomic selection may be a promising strategy for continuing to improve forage digestibility [44]. Genomic selection models also show that separating tissue types enhances accuracy, and prediction accuracy is higher for the stem NDFD than for leaf NDFD [44].

Genome-wide markers make it possible to quantify additive and non-additive gene effects. Parametric genomic selection (GS) models are known to capture additive genetic effects but are not efficient with epistatic effects due to the computational burden of high-order interactions [46,47]. Semi-parametric and non-parametric GS models capturing epistatic interactions have been developed and implemented, including high-order interactions in plant breeding [48,49]. Due to the high heritability for 96 h IVNDFD and the potential to capture epistasis and dominance, the use of genomic prediction for stem digestibility with the new non-parametric GS models might further improve breeding efforts to enhance alfalfa forage digestibility. The populations developed in this study will be valuable resources in future experiments to develop GS methods to accelerate breeding for fiber digestibility and to further dissect the morphological and/or compositional changes in alfalfa stems in response to selection.

5. Conclusions

Recurrent selection for alfalfa stem IVNDFD was a successful strategy for improving fiber digestibility and reducing lignin in stems without changing the proportion of leaves to stems in total herbage. Increased digestibility and decreased lignin occurred in later maturity stages, which would increase biomass yields while maintaining forage quality. The selection also reduced seasonal variability in stem digestibility. Successive selection cycles changed the environmental stability of IVNDFD and ADL/NDF, indicating that these traits should be evaluated in multiple environments when developing alfalfa with improved nutritional value. If the mode of action for increased IVNDFD is found to be different than current genetically modified reduced lignin lines, there is a potential for stacking improved stem digestibility traits.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy13030770/s1: Figure S1: Environmental stability of 16 h IVNDFD with selection cycles across the 12 harvest environments. (Left) H × H, intermating of plants with high 16 h IVDFD and high 96 h IVNDFD. (Right) L × L, intermating of plants with low 16 h IVDFD and low 96 h IVNDFD. Table S1: Calibration statistics for near-infrared reflectance spectroscopy (NIRS) prediction equations for in vitro neutral detergent fiber digestibility (IVNDFD), detergent fiber components, and CW traits of alfalfa stems; Table S2: p-values of the 5-way analysis of variance (ANOVA) for 16 h IVNDFD (NDFD16), 96 h IVNDFD (NDFD96), acid detergent lignin as a proportion of NDF (ADL), neutral detergent fiber (NDF), stem dry weight (SDW), and percent stems (Per_stem) among the following five variables: year (Y), location (L), germplasm (G), maturity (M), and harvest (H); Table S3: Summary of 16 h IVNDFD (NDFD16H), 96 h IVNDFD (NDFD96H), acid detergent lignin as a proportion of NDF (ADL), neutral detergent fiber (NDF), stem dry weight (SDW), and percent stems (Per_stem) of alfalfa populations across years, locations, harvests, and maturities. C0, unselected population; C1, Cycle 1 population; C2, Cycle 2 population; H × H, H16 × H96, intermating of plants with high 16 h IVDFD and high 96 h IVNDFD; L × L, L16 × L96, intermating of plants with low 16 h IVDFD and low 96 h IVNDFD. NDF is on a dry matter (DM) basis. ADL is on an NDF basis. Table S4: Change in ADL in the H16 × H96 populations with cycle of selection at the early bud (EB), late flowering (LF), and green pod (GP) maturity stages over three harvests. LSD is Fisher's least significant difference test with different letters within a maturity stage indicating a significant difference; %/cycle is the percent change from C0 to C1 and C1 to C2. Table S5: Summary of stem dry weight (SDW) and herbage dry weight (HDW) changes among maturities, harvests, and selection cycles. LSD is Fisher's least significant difference (LSD) test with different letters within a maturity stage indicating a significant difference; %/cycle is the percent change from C0 to C1 and C1 to C2.

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