CHANGES IN ALFALFA CELL WALL STRUCTURE DURING VEGETATION

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Abstract: The investigation was done on 141 samples of one alfalfa cultivar, collected from the same location during the first three growth cycles: spring growth, the first and the second regrowth. Within each growth cycle, sampling was done during the whole growing period, commencing when plant height was below 150 mm and continuing until plants were bearing ripe seeds. On all collected samples the following cell wall characteristics were determined: neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), neutral detergent insoluble crude protein (NDICP), acid detergent insoluble crude protein (ADICP). Cellulose and hemicellulose were detected on the base of the mentioned chemical parameters. Significantly lower ($p<0.01$) content of aNDF, ADF, ADL, ADICP and cellulose is found in the second regrowth, while there were no significant differences between the other two growth cycles. Except in NDICP and ADICP, the increase in all accompanying components of the cell wall was observed, and expressed in average daily changes. There was no consistent trend in NDICP and ADICP. During the spring growth from late bud to full-bloom stage the 'plateau' was observed. The plateau was represented as almost constant content of aNDF, ADF, ADL and cellulose. The correlations between all components of the cell wall were shown. The equation aNDF = 36.713 + 1.181 × ADF is recommended for conversion of ADF into aNDF in alfalfa.

Key words: growth cycle, neutral detergent fibre, acid detergent fibre, acid detergent lignin, crude protein linked to cell wall.

Introduction

In all climate regions where alfalfa (*Medicago sativa* L.) grows, it is the dominant forage culture because of its high nutritive value. It is of the utmost importance in the ruminant nutrition where it is mostly used as hay, silage or

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haylage. In dairy cow nutrition as forage fodder, it has to supply the adequate amount of fiber in the ration. On the other hand, the increased amount of fiber in alfalfa can reduce its nutritive value and decrease animal intake (Van Soest, 1994). This is the reason why the understanding of the changes in alfalfa composition during its vegetation is crucial for its successful use in the intensive dairy cow production. The conversance of the changes that occur in the cell wall components during the vegetation can lead to better utilization and to adequate estimation of the optimal moment for alfalfa cutting. The available data mostly show the average values of cell wall components in the various development phases of alfalfa (DLG, 1997; NRC, 2001; Thomas, 2004; INRA, 2007), while detailed information about their changes during the whole vegetation is not usually shown. The dynamics of changes in cell wall components in connection with alfalfa mean stage of development are presented in numerous articles (Kalu and Fick, 1983; Fick and Onstad, 1988; Sanderson and Wedin, 1988; Hintz and Albrecht, 1991; Griffin et al., 1994.).

The aim of this investigation is to evaluate the changes in cell wall components that occur during the alfalfa vegetation in the first three growth cycles. The changes in cell wall are investigated as transformations of cell wall components, and the connections between them are established.

Material and Methods

The detailed sampling procedure was given by Božičković et al. (2012; 2013). During 2010, on the same alfalfa field a total of 141 fresh samples were collected. The trial was confined to a single cultivar, Banat, which was sown in the spring of 2008. Sampling was carried out during three growth cycles: spring growth, the first regrowth and the second regrowth. Within each growth cycle, sampling was done during the whole growing period, commencing when plant height was below 150 mm and continuing until plants were bearing ripe seeds. The samples were obtained by hand clipping a randomly selected area of 0.12 m^2. Clipping was done at the height of 3.5 cm, and all shoots with stems above clipping height were collected. The description of sampling dynamics is shown in Table 1.

After clipping, the samples were placed in plastic bags, stored in a portable refrigerator, and transported to the laboratory for processing. In the laboratory, by inspection of all shoots in collected samples, the presence and number of shoots with buds, flowers and green pods were observed. Samples were coarsely chopped into separate bags, immediately frozen to -18°C, and stored at this temperature for at least 3 days before further processing. After that, samples were dried in a forced air oven (60°C).

After drying, samples were ground to pass through a 1 mm sieve, and they were stored in plastic bags at 4°C until chemical analysis was performed. The

following analyses were conducted: neutral detergent fibre (aNDF), acid detergent fibre (ADF), acid detergent lignin (ADL), neutral detergent insoluble crude protein (NDICP) and acid detergent insoluble crude protein (ADICP). All analytical results were expressed on a dry matter (DM) basis, obtained by drying at 103°C until constant weight was achieved, which was 4±0.1 h as defined by ISO (1999).

Table 1. Sampling dynamics during 2010.

Growth cycle	Beginning of growth cycle	Sampling		Number of sampling sessions	Number of samples
		The first	The last		
Spring growth	21 March	7 April	15 July	33	72
The first regrowth	24 May	10 June	18 July	15	35
The second regrowth	1 July	6 July	9 August	13	34
Total				61	141

The Gerhardt FibreBag (C. Gerhardt GmbH & Co., Königswinter, Germany) apparatus was used to analyze aNDF, ADF and ADL. Neutral detergent fibre was analyzed with the use of 50 µl of amylase (Sigma A 3306, Sigma-Aldrich, St. Louis, MO, USA) without sodium sulphite according to Van Soest et al. (1991). Acid detergent fibre and ADL were analyzed according to ISO (2008). The aNDF and ADF analyses were not done sequentially and were expressed inclusive of residual ash. NDICP and ADICP were detected with the analysis of crude protein in aNDF and ADF. Crude protein was analyzed according to Kjeldahl (ISO, 2005), using the Tecator Kjeltec Auto Analyzer 1030 (Kjeltec, Tecator AB, Höganäs, Sweden).

Cellulose content was determined using equation Eq. (1) while hemicellulose was determined using Eq. (2). This procedure for cellulose and hemicellulose calculation was used to provide the insight into changes within aNDF connected to alfalfa maturing. The problems of such nonsequential analysis are presented by pectin and silica (Van Soest et al., 1991). Pectin and silica are soluble in neutral detergent and not soluble in acid detergent. For that reason, the calculated hemicellulose is underrated because pectin and silica remain in the ADF, while cellulose is somewhat overrated.

$$\text{Cellulose} = \text{ADF} - \text{ADL} - \text{ADICP} \qquad\qquad \text{Eq. (1)}$$

$$\text{Hemicellulose} = (\text{aNDF} - \text{NDICP}) - (\text{ADF} - \text{ADICP}) \qquad \text{Eq. (2)}$$

All statistical analyses were performed using the software package Statistica 6 (Statsoft, Inc., 2003).

Results and Discussion

The average values of all investigated cell wall parameters are given in Table 2, with their variations within each growth cycle. Somewhat higher minimal amounts of the investigated parameters in the first regrowth are the result of the fact that starting samples were collected a bit later. Bad weather conditions in that part of the year postponed the collecting of samples in that growth cycle. With the exclusion of NDICP and hemicellulose, the second regrowth had significantly lower values for all cell wall components ($p<0.01$). The second regrowth lasted throughout the hottest part of the year (Table 1) which is a deviation from literature (Griffin et al., 1994; Van Soest, 1994; Komprda et al., 1997).

Table 2. Average values of investigated parameters of nutritive value in alfalfa within different growth cycles and for all of them together. All parameters are expressed in g/kg of dry matter (DM). Standard deviation (SD) and min-max interval are given in parentheses.

Parameter	Growth cycle			
	Spring growth (n=72)	The first regrowth (n=35)	The second regrowth (n=34)	All growth cycles[1] (n=141)
aNDF	426.56[B]	445.41[B]	375.55[A]	418.94
	(82.32; 234.32-558.28)	(56.52; 279.36-525.26)	(58.15; 228.7-447.49)	(75.31; 228.7-558.28)
ADF	327.68[Bb]	357.02[Ba]	280.70[Aab]	323.64
	(65.35; 161.26-433.54)	(45.68; 236.71-409.4)	(45.94; 154.76-334.98)	(62.49; 154.76-433.54)
ADL	64.05[B]	65.69[B]	54.37[A]	62.12
	(18.93; 21.6-103.55)	(15.59; 34.79-86.95)	(11.29; 22.72-70.61)	(17.04; 21.6-103.55)
NDICP	31.59[B]	27.59[A]	31.64[B]	30.61
	(5.1; 25.17-51.17)	(3.43; 20.37-36.24)	(6.95; 24.08-54.89)	(5.52; 20.37-54.89)
ADICP	9.97[B]	10.16[B]	9.02[A]	9.79
	(1.85; 6.26-14.98)	(1.13; 8.27-12.43)	(1.42; 6.4-13.32)	(1.65; 6.26-14.98)
Cellulose	253.67[C]	281.17[B]	217.31[A]	251.73
	(46.17; 132.59-324.41)	(30.97; 192.11-321.83)	(34.47; 125.64-256.84)	(45.83; 125.64-324.41)
Hemicellulose	77.25	70.96	72.23	74.48
	(21.18; 32.09-119.36)	(13.48; 31.85-100.1)	(17.85; 26.57-105.94)	(18.84; 26.57-119.36)

[A,B,C] Average values of separate growth cycles with a different letter are significantly different (LSD test; $p<0.01$). [a,b,c] Average values of separate growth cycles with a different letter are significantly different (LSD test; $p<0.05$). [1] Average values calculated for all growth cycles were not compared with averages for separate growth cycles. aNDF – neutral detergent fibre; ADF – acid detergent fibre; ADL – acid detergent lignin; NDICP – neutral detergent insoluble crude protein; ADICP – acid detergent insoluble crude protein.

The digestibility of forages is in a negative correlation with the content of lignin and all cell wall components. According to Van Soest (1994) high daily temperatures increase lignification while decrease digestibility. However, in alfalfa, at the same time, due to the increase of light and day length, there is a

change in leaf: stem ratio in favor of leaves, and because of that the decrease in digestibility can be slowed down. This is just a part of the explanation why the statistically significant lower amounts of almost all cell wall components were observed in the second regrowth. With the exception of ADF and cellulose between spring growth and the first regrowth, there were no statistically significant differences ($p>0.05$). The lower content of NDICP in the first regrowth most probably occurred because sampling was done somewhat later in this growth cycle.

Based on the observation of shoots in all samples, the following phases were detected: early bud, late bud, early bloom and full-bloom (Table 3). The shown intervals were detected on the basis of relative ratio of shoots with buds and flowers to the total number of shoots within one sample. In practical conditions with this phase of alfalfa development, the optimal moment for harvesting is determined, while early bud represents very early cutting.

Table 3. The intervals of the most significant moments during the investigated growth cycles, shown in days of vegetation. Dates are given in parentheses.

Growth cycle	Early bud	Late bud	Early bloom	Full-bloom
Spring growth	44 – 46 (4–6 May)	48 – 54 (8–14 May)	55 – 59 (15–19 May)	63 – 67 (23–27 May)
The first regrowth	17 – 20 (10–13 June)	22 – 26 (15–19 June)	28 – 31 (21–24 June)	35 – 37 (28–30 June)
The second regrowth	11 – 14 (12–15 July)	17 – 22 (18–23 July)	23 – 24 (24–25 July)	28 – 29 (29–30 July)

The changes in cell wall parameters are shown in Figure 1. Aside from NDICP and ADICP, all other parameters had increasing trends with the advancing of the vegetation. In the middle of the spring growth, aNDF, ADF, ADL, cellulose and hemicellulose had no clear trend. Considering the explained intervals (Table 3), it can be concluded that from late bud to full-bloom there was almost constant level of aNDF, ADF and ADL in alfalfa. Also, during the early bud phase, alfalfa had an average of 419 g/kg SM aNDF and 324 g/kg SM ADF. According to Buxton (1996), alfalfa hay with such a composition would be considered as 'fair category'. However, due to the loss in leaves during the hay making, the hay from this material would probably have more ADF and aNDF, and therefore it would be classified as 'low category' hay. Considering that harvesting in the early bud stage would be very early, the quality of such hay would not be acceptable. Because of all that, the adequate evaluation of optimal moment for cutting can be conducted only with the data about chemical composition and nutritive value of alfalfa. For that estimation, different methods can be utilized: mean stage of development (Kalu and Fick, 1981; Božičković et al., 2013) and method according to Hintz and Albrecht (1991). It is also possible to use the Near-infrared spectroscopy (NIRS).
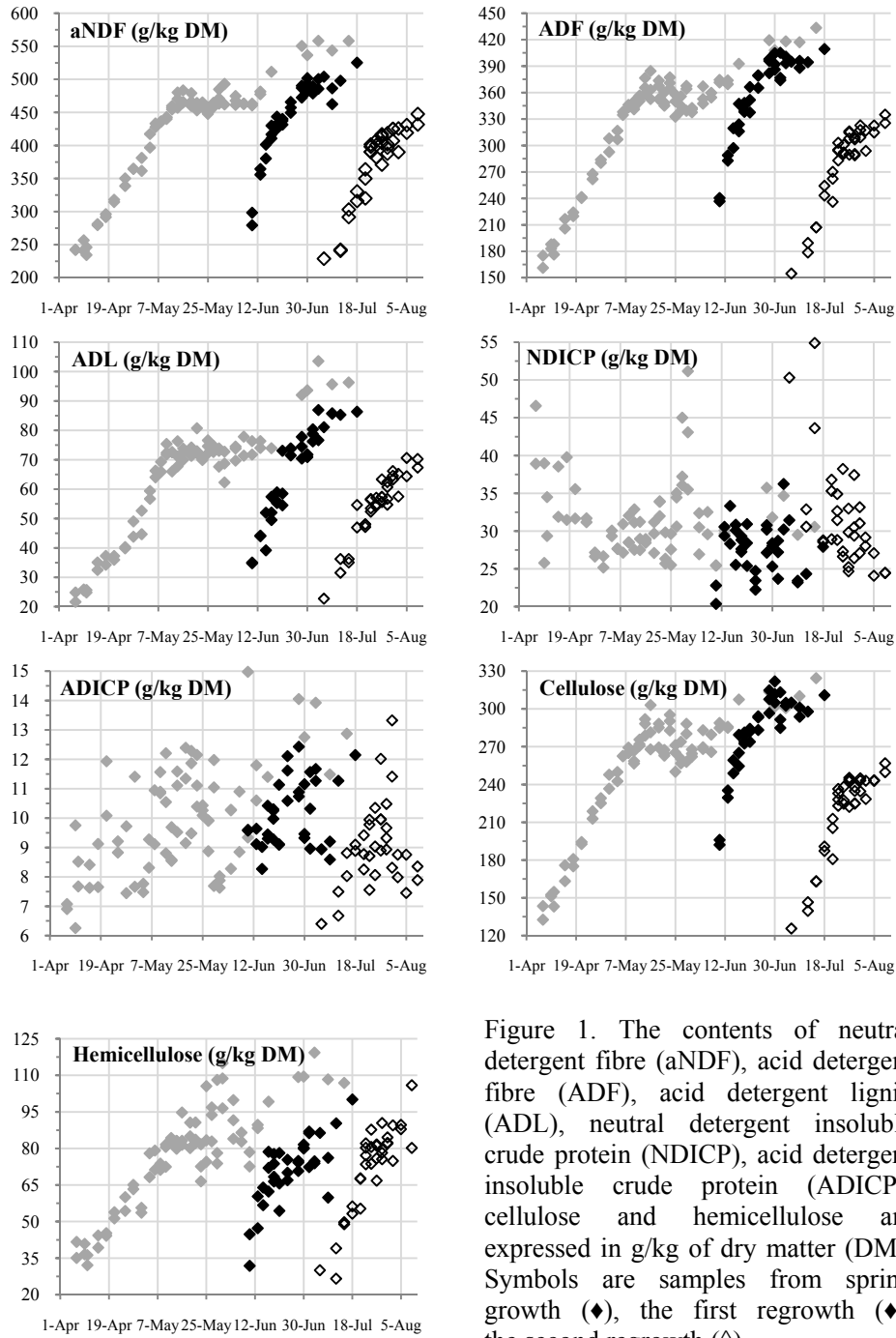
Figure 1. The contents of neutral detergent fibre (aNDF), acid detergent fibre (ADF), acid detergent lignin (ADL), neutral detergent insoluble crude protein (NDICP), acid detergent insoluble crude protein (ADICP), cellulose and hemicellulose are expressed in g/kg of dry matter (DM). Symbols are samples from spring growth (♦), the first regrowth (♦), the second regrowth (◊).

The trend observed in crude protein (CP) within the same samples is shown by Božičković et al. (2012). Similarly to the fractions of cell wall, during the budding phase, the content of CP had the lowest level of about 170 g/kg DM, which was not changed until the end of the spring growth. According to McDonald et al. (2011), digestibility decreases as plants increase in maturity, but the relationship is complicated by there being a spring period of up to a month during which the herbage digestibility remains fairly constant. This period has been described as the 'plateau'. Considering that plateau was observed in cell wall fractions and also in CP, it can be concluded that the digestibility would follow the same pattern, and that, in this period, the lowest level of digestibility was reached. This was confirmed by *in vitro* investigation of organic matter digestibility, which was rather constant during that period at the level of about 650 g/kg of organic matter (Božičković, unpublished). The observed level of organic matter digestibility is characteristic for alfalfa at the end of flowering, according to DLG (1997).

On the basis of the obtained results, the linear regression equations were derived, which determine the daily changes in cell wall components investigated (Table 4). In the first and the second regrowths, the daily changes were obtained based on regression functions given for the whole investigating period. On the other hand, in the spring growth, linear regression functions were derived only for the period from the beginning of vegetation until the plateau was reached, which was the 56th day of vegetation (16 May 2010) for aNDF, ADF, cellulose and hemicellulose, while it was the 50th day of vegetation (10 May 2010) for ADL. Because of the high randomization of data, functions were not derived for NDICP and ADICP. The slowest daily changes in all parameters were observed in the first regrowth. This is due to the fact that the trend in changes in all cell wall parameters in the first regrowth had quadratic form, and therefore the linear equations had lower slope. This is why the coefficient of determination (R^2) in all linear equations for the first regrowth was the lowest.

Table 4. Daily changes in investigated cell wall components.

Growth cycle	g/kg DM daily				
	aNDF	ADF	ADL	Cellulose	Hemicellulose
Spring growth	6.47	5.38	1.48	3.90	1.37
The first regrowth	5.12	4.13	1.52	2.57	1.04
The second regrowth	6.64	5.27	1.33	3.88	1.99

DM – dry matter; aNDF – neutral detergent fibre; ADF – acid detergent fibre; ADL – acid detergent lignin.

The coefficients of correlations between all investigated cell wall parameters are given in Table 5. The shown coefficients were calculated by summarising all

investigated vegetation cycles, based on all 141 samples. Very strong positive correlations between aNDF, ADF, ADL and cellulose were determined, while hemicellulose was strongly correlated with cell wall parameters. On the other hand, NDICP had a very weak correlation with all other parameters. ADICP had a moderate correlation with the other cell wall parameters and a weak correlation with NDICP.

Table 5. Correlation coefficients between investigated cell wall parameters, calculated based on all collected samples (n=141).

	aNDF	ADF	Cellulose	Hemicellulose	ADL	NDICP	ADICP
aNDF		0.9799	0.9612	0.8670	0.9532	0.2404	0.5659
ADF	0.9799		0.9941	0.7603	0.9408	0.3640	0.6080
Cellulose	0.9612	0.9941		0.7173	0.8902	0.3861	0.5809
Hemicellulose	0.8670	0.7603	0.7173		0.8190	0.0693	0.4088
ADL	0.9532	0.9408	0.8902	0.8190		0.2713	0.5704
NDICP	0.2404	0.3640	0.3861	0.0693	0.2713		0.2598
ADICP	0.5659	0.6080	0.5809	0.4088	0.5704	0.2598	

aNDF – neutral detergent fibre; ADF – acid detergent fibre; ADL – acid detergent lignin; NDICP – neutral detergent insoluble crude protein; ADICP – acid detergent insoluble crude protein.

The shown trend in ADL growth and lack of trend in NDICP and ADICP had weak and moderate correlations between those parameters as a consequence. This is different from the results of some earlier investigations (Sniffen et al., 1992; Van Soest 1994), where there was a strong connection between lignin and ADICP content. Considering the shown interval of ADL and ADICP variations, the five-fold increase in ADL was followed with a two-fold increase in ADICP content.

The derivations of equations for conversion of ADF to aNDF are justified considering that NDF analysis is much more expensive than ADF analysis, which may be the reason why in some countries detergent analysis is restricted to ADF only (Van Soest, 1994). The linearity of this connection can be assumed based on the high correlation coefficient (Table 5), and it is shown in Figure 2.

The Eq. 3, shown on Figure 2, was derived on the basis of all samples (n=141). The exactness of this equation is shown with its coefficient of determination (R^2) and with root mean square error (RMSE). R^2 is percent of aNDF variability, which can be explained with ADF, while RMSE represents an average deviation of the derived regression from the values observed in collected samples. Consequently, the error of this equation is 14.95 g/kg DM aNDF. This error is maximally 7% of aNDF in early samples with low aNDF content, while in later ones, with aNDF of 300 to 450 g/kg DM, it ranged from 2% to 3%. Similar

equations derived mostly for alfalfa were given by Putnam (1998, 2004) and Putnam and Undersander (2006), but their equations had lower R^2 than Eq. (3). The equations by the aforementioned authors were obtained based on alfalfa hay, which is somewhat altered plant material compared to the material used in this investigation.
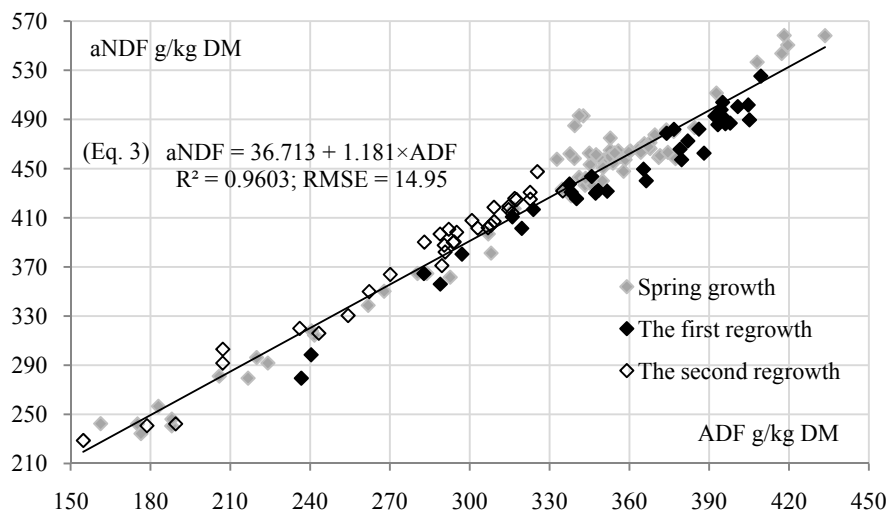

Figure 2. The connection of acid detergent fibre (ADF) and neutral detergent fibre (aNDF) contents in all collected samples (*n*=141). ADF and aNDF contents are expressed in g/kg of dry matter (DM).

Conclusion

This investigation confirmed that there are differences in the cell wall composition between different growth cycles of alfalfa. Regarding the obtained results, the lowest content of aNDF, ADF, ADL, ADICP and cellulose (*p*<0.01) was observed in the second regrowth. In the spring growth from late bud to full-bloom phase, the plateau was observed in all investigated parameters of the cell wall, except in hemicellulose, NDICP and ADICP. Further investigations are required to determine if those phenomena are the results of agroecological conditions during conducted investigation, or they are characteristics for wider area of Serbia. The fractions of proteins connected to cell wall, NDICP and ADICP, showed high variability and a weak connection with other components of the cell wall composition. The derived equation for conversion of ADF into aNDF had deviation between 2% and 7% from the calibration set of data.

Acknowledgements

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PROMENE U STRUKTURI ĆELIJSKOG ZIDA LUCERKE TOKOM VEGETACIJE

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R e z i m e

Istraživanje je sprovedeno na 141 uzorku iste sorte lucerke, sakupljene na istoj lokaciji tokom prva tri ciklusa vegetacije: prolećni ciklus, drugi ciklus i treći ciklus. Tokom svakog ciklusa, uzorkovanjem je obuhvaćen ceo ciklus vegetacije, od momenta kada su biljke imale visinu manju od 150 mm sve do momenta kada su biljke imale zrelo seme. U svim sakupljenim uzorcima određeni su sledeći parametri ćelijskog zida: vlakna nerastvorljiva u neutralnom deterdžentu (aNDF), vlakna nerastvorljiva u kiselom deterdžentu (ADF), lignin (ADL), protein nerastvorljiv u neutralnom deterdžentu (NDICP), protein nerastvorljiv u kiselom deterdžentu (ADICP). Celuloza i hemiceluloza su određene na osnovu navedenih hemijskih parametara. Utvrđena je značajno manja ($p<0.01$) količina aNDF, ADF, ADL, ADICP i celuloze u trećem ciklusu vegetacije, dok između ostala dva ciklusa nisu utvrđene veće razlike. Izuzev kod NDICP i ADICP, utvrđen je porast svih praćenih komponenata ćelijskog zida i izražen u prosečnim dnevnim promenama. Kod NDICP i ADICP je zabeleženo odsustvo trenda. Tokom prolećnog ciklusa od faze punog pupoljenja do faze punog cvetanja je zabeležen 'plato', odnosno skoro konstantna količina aNDF, ADF, ADL i celuloze. Prikazane su korelacije između svih parametara ćelijskog zida. Jednačina aNDF = 36.713 + 1.181 × ADF je preporučena za konverziju ADF u aNDF u lucerki.

Ključne reči: vegetacioni ciklus, vlakna nerastvorljiva u neutralnom deterdžentu, vlakna nerastvorljiva u kiselom deterdžentu, lignin, protein vezan za ćelijski zid.

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