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CHANGES IN AGRONOMIC AND FORAGE NUTRITIVE VALUES OF RED CLOVER IN RESPONSE TO DIFFERENT DEVELOPMENT STAGE

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ABSTRACT

Red clover (*Trifolium pratense* L.) is the second most important cultivated perennial forage legume used as a fodder for livestock. Agronomic and forage quality traits of red clover are dependent on various factors, including optimal phenological stage for cutting in order to obtain forage with high nutritive value and yield. Three different development stages (full budding, beginning of flowering, full flowering) of two red clover genotypes were examined in order to determine changes in agronomic and forage nutritive values. The stage of plant development at the time of cutting significantly affected yields and forage nutritive values of red clover compared to the choice of cultivar. Forage cut at full flowering resulted in the highest values of green mass, dry matter yield and crude protein yield and plant height, however the forage quality with the plant maturation rapidly decreased. The correlations between the investigated traits confirmed a negative relationship between yields and forage nutritive quality.

Keywords: cutting, development stage, forage quality, nutritive value, red clover.

INTRODUCTION

Forage crops are an important livestock feed resource and play a significant role in animal production and livestock farming systems around the world. Perennial forage legumes used as a base feed are excellent fodder for many classes of livestock including dry dairy cows, dairy heifers, dairy beef, or beef cows (Lüscher et al., 2014; Hanson and Ellis, 2020). Red clover (*Trifolium pratense* L.) has a long history of relevance in Europe, and is after alfalfa (*Medicago sativa* L.) the second most important cultivated perennial forage legume based on the amounts of seed produced and marketed, and on cultivar availability (Boller et al., 2010; Hejduk and Knot, 2010; Hoekstra et al., 2018). This perennial plant offers several advantages such as high protein content and soil improving characteristics, which reduce the use of artificial nitrogen fertilizers and enhance livestock feed intake (McKenna et al., 2018; Herbert et al., 2021; Przybylska et al., 2021).

Red clover can be grown in monoculture or more frequently in mixtures with grasses such as timothy (*Phleum pratense* L.), meadow fescue (*Festuca pratensis* Huds.), tall fescue (*Festuca arundinacea* Schreb.) and/or perennial ryegrass (*Lolium perenne* L.) for hay, haylage, silage or grazing (Amdahl et al., 2016; Ergon et al., 2019; Naydenova and Vasileva, 2019; Moloney et al., 2020; Egan et al., 2021). This species is known to cope well in a wide range of soil types, pH levels, and

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environmental and management conditions (Surmen et al., 2013; Ortega et al., 2014; Chmelikova et al., 2015; Tucak et al., 2019; López Olivari and Ortega Klose, 2020). Red clover is a short-lived perennial and in a temperate climate of Southeast Europe its production normally persists from two to four years with few cuts. In one growing season red clover is capable of producing yields in the range of 40-80 t ha^{-1} year^{-1} of green mass and of 8-18 t ha^{-1} year^{-1} of dry matter. In addition to the high yielding capacity, red clover has good forage quality and high nutritive value of hay.

Agronomic and forage quality traits are highly complex in nature. Their expression is affected by many independent factors, as well as their relationships, including genetic background (cultivars), environmental conditions (especially drought stress through direct and indirect effects on plant morphology and physiology, soil fertility), cutting frequency, phenological stages of development, cutting height, harvest and storage, as well as growing year (Drobna and Jančović, 2006; Kleen et al., 2010; Atis et al., 2019; Nadeem et al., 2019; Petcu et al., 2019; Wróbel and Zielewicz, 2019; Katanski et al., 2020; Marković et al., 2021; Tucak et al., 2021).

Determination of the optimal phenological stage for cutting is of the most importance for obtaining red clover forage with high nutritive value as well as meeting yield requirements. Cutting is the main agronomic factor that affects morphology and the expression of yield potential, and indirectly determines nutritive value (Fracchiolla et al., 2018). Red clover cut at bud stage will have highest forage quality (the highest concentration of crude protein and lowest concentration of neutral and acid detergent fiber, high proportion of leaves), but also this results in lower yield per cut. On the other hand, cutting red clover in the later phenological stages of development results in a significant forage quality decrease in the sense of increased fiber, declined crude protein, and decreased digestibility within red clover herbage. Therefore, cutting management of perennial legumes such as red clover and alfalfa requires a compromise between quality and yield.

The objective of this study was to characterize changes of the red clover productivity and forage nutritive value throughout three different development stages and to investigate correlations between the studied traits.

MATERIAL AND METHODS

Study site, crop establishment and experimental design

Research was conducted at the experimental field of the Agricultural Institute Osijek, Eastern Croatia, during the 2018 growing season. The soil type of the site was eutric cambisol of neutral pH reaction. The climate of the experimental site is characterized by an annual precipitation sum of 654.2 mm and an average annual temperature of 10.9°C (1970-2000, Source: Meteorological and Hydrological Service of the Republic of Croatia).

The experiment was designed as a two factorial trial in a randomized complete block design with three replicates. Red clover was sown on 15 March 2017, with a seeding rate of 18 kg ha^{-1}. Individual plot size was 24 m^2, consisting of 12 rows, each 10 m in length with between row spacing of 20 cm. Irrigation, fertilization or weed/pest/disease protection were not applied. Three different development stages (full budding on May 8, beginning of flowering on May 18, full flowering at the end of May) of two red clover genotypes (cultivar OS VIVA and breeding population CD-3) were examined in the first cut in the second year of growth.

Agronomic data measurement and forage samples collection

In each studied development stage on all plots plants height (PH, cm) was measured on 10 randomly selected red clover plants from the middle row of the experimental plot. Average samples of approximately 500 g of forage green mass were collected from the middle of each plot in each development stage, weighed fresh, dried in a dryer at 105°C for 48 h and weighed dry to determine dry matter content in order to calculate dry matter yield (DMY). The values were converted into DMY in t ha^{-1}. For the quality analysis, fresh herbage samples were taken from each plot in all development stages, dried at 60°C for 48 h and were ground to pass through a 1 mm screen using a Retsch ultracentrifugal mill (Type ZM1, Haan, Germany). Ground forage samples were sent to the Animal Science Department laboratory for NIR spectroscopy at the Agricultural Institute of Slovenia, where the analysis was performed.

Green mass yield (GMY) was determined by cutting the whole plot using a forage plot harvester with an electronic weigh system (Hege Model 212, Wintersteiger AG, Germany) and then the data obtained was also converted to t ha^{-1}. Crude protein yield (CPY) was obtained from the value of crude protein content (CP) and dry matter yield, and converted into kg ha^{-1}.

Forage nutritive value analyses

Forage quality measurements such as the content (%) of crude protein (CP), acid detergent fiber (ADF), neutral detergent fiber (NDF), and total digestible nutrients (TDN) were analysed using a NIRSystems 6500 Monochromator (Foss NIRSystem, Silver Spring, MD). All analytical results were expressed on a dry matter (DM) basis.

Digestibility of dry matter (DDM), dry matter intake (DMI), relative feed value (RFV), net energy-lactation (NEL), net energy-maintenance (NEm) and net energy-gain (NEg) were calculated according to the following equations adapted from common formulas for forages (Schroeder, 1994):

$$DDM\ \% = 88.9 - (0.779 \times ADF\ \%),$$

$$DMI\ \% = 120 / NDF,$$

$$RFV = (DDM\ \% \times DMI\ \%) / 1.29,$$

$$NEL = 1.044 - (ADF\% \times 0.0123),$$

$$NEm = (TDN\ \% \times 0.01318) - 0.132,$$

$$NEg = (TDN\ \% \times 0.01318) - 0.459.$$

The energy values were expressed in MJ kg^{-1} DM.

Statistical Analysis

All obtained agronomic and forage nutritive data were subjected to a two factorial analyses of variance (ANOVA), with development stages and cultivars as factors, using STATISTICA Statistical Software Package v8.0 (StatSoft, Inc., Tulsa, OK, USA). Where F-tests were significant ($p<0.01$), Least Significant Difference (LSD) test at 0.05 level of probability was calculated to compare means. Correlation between investigated parameters was determined by Pearson correlation coefficients (r).

RESULTS AND DISCUSSION

Development stage and cutting time are certainly one of the major factors determining quality and quantity parameters of the forage crops (Marković et al., 2011; Testa et al., 2011; Min, 2016; Monirifar et al., 2020). The results of ANOVA showed statistically significant differences between different development stages for all the investigated agronomic and forage quality traits in red clover cultivar/population (Table 1). Statistically significant differences between cultivars were obtained for CP content and CPY, and ADF and NDF content values, while the cultivar did not have a significant influence on the other observed properties. The development stage × cultivar interaction for most of the studied traits was not significant, except for GMY and ADF.

Table 1. Analysis of variance (ANOVA) for the investigated agronomic and forage quality traits in red clover cultivar/population over the three development stages

Source of Variation	DF	Traits					
		GMY	DMY	CPY	PH	CP	ADF
		F-value					
DS	2	118.17**	4.15**	24.95**	520.94**	25.34**	271.1**
C	1	0.06ns	0.12ns	15.36**	4.20ns	2.20**	516.0**
DS × C	2	8.05**	0.02ns	2.30ns	1.04ns	0.46ns	103.1**
Source of Variation	DF	Traits					
		NDF	RFV	TDN	NEL	NEm	NEg
		F-value					
DS	2	82.50**	3789.8**	30.68**	0.63**	0.45**	0.45**
C	1	3.56**	120.2ns	0.20ns	0.04ns	0.03ns	0.03ns
DS × C	2	0.67ns	16.4ns	0.28ns	0.05ns	0.04ns	0.04ns

Note: DF - degrees of freedom, DS - development stage, C - cultivar, DS × C - interaction development stage × cultivar, **significance level at $P \leq 0.01$, ns - not significant.

The highest values of GMY, DMY, CPY and PH were found in the third development stage (Table 2). In this development stage average values of GMY, DMY and PH (27.93 t ha^{-1}, 5.17 t ha^{-1} and 66.15 cm) were for about 29% higher compared to the lowest values achieved in the first development stage. The lowest CPY was in the second development stage, which was for about 14% lower than the CPY obtained in the third development stage (807.6 kg ha^{-1}). Differences between cultivar OS VIVA and breeding population CD 3 in yields and plant height were not significant, however the population proved to be superior, as it had higher values for all traits in all development stages as well as for averages of all stages, except for GMY and DMY in the third development stage. Albayrak and Turk (2013) evaluated changes in the forage yield and quality of legume-grass mixtures throughout a vegetation period and by harvesting the red clover at the late vegetative stage in first cut of the second year of research determined DM yield of 5.20 t ha^{-1}. Norman et al. (2021) tested productivity and nutritional value of 20 species of perennial legumes over two years, and in red clover cultivars cut in different growth stages in the second year after sowing found variation for DM yield from 4 t ha^{-1} to 5.7 t ha^{-1} (development stage bud to first flower). Similar findings were found in present study. Research by numerous authors about the effects of cutting stages on yield and quality of different forage crops noted that early cutting resulted in low yield with high protein content and digestibility, while late harvest lead to high yield with low protein content and digestibility (Wiersma et al., 1998; Testa et al., 2011; Albayrak et al., 2013; Fracchiolla et al., 2018), which this study will present in the following results.

Table 2. Agronomic traits of the red clover cultivar/population during different development stage (DS) in the first cut of the second year of growth 2018

Traits	GMY (t ha^{-1})			DMY (t ha^{-1})		
Cultivar/Mean	VIVA	CD-3	Mean	VIVA	CD-3	Mean
I DS	19.54	20.04	19.79	3.57	3.73	3.65
II DS	19.70	21.91	20.81	3.67	3.95	3.81
III DS	29.12	26.75	27.93	5.14	5.19	5.17
Mean	22.79	22.90		4.13	4.29	
LSD 0.05ds	0.94			0.24		
LSD 0.05c	ns			ns		
LSD 0.05ds × c	1.33			ns		
Traits	CPY (kg ha^{-1})			PH (cm)		
Cultivar/Mean	VIVA	CD-3	Mean	VIVA	CD-3	Mean
I DS	681.9	757.2	719.1	47.40	47.70	47.55
II DS	648.1	729.6	688.5	55.50	56.20	55.85
III DS	799.8	813.3	807.6	65.20	67.10	66.15
Mean	720.3	778.2		56.03	57.00	
LSD 0.05ds	37.77			1.41		
LSD 0.05c	30.84			ns		
LSD 0.05ds × c	ns			ns		

Note: I DS of budding, II DS of beginning of flowering, III DS of full flowering.

The CP, ADF, NDF contents in DM are one of the most important traits which provide useful information on forage quality. The results of this research indicated that CP content decreased from the first development stage (19.70%) to the third development stage (15.62%). On the other hand, during maturation the ADF and NDF content significantly increased and ranged from 26.41% (budding) to 31.70% (full flowering) for ADF, and 30.93% (budding) to 38.17% (full flowering) for NDF, respectively (Table 3). Breeding population CD 3 in relation to cultivar OS VIVA achieved higher mean CP content (18.14%) and had more favorable values at individual stages of development. Population also had a slightly higher NDF content compared to the cultivar. Red clover leaves are the main source of protein and amount of leaves in the total herbage mass is the largest in the earlier stages of development. During plant maturation a decrease in leaf and increase in stem proportion and the process of lignification all result in a decrease in forage quality and digestibility. Similar trend of changes of chemical composition in perennial and annual clovers herbage during cutting through different development stages was previously determined by Vasiljević et al. (2009), Marković et al. (2011), Albayrak et al. (2013), Fracchiolla et al. (2018) and Wróbel and Zielewicz (2019).

RFV was affected by development stage of plant, while differences among cultivar/population for RFV were not determined (Table 3). The highest RFV was recorded in the first (205.71), and the lowest in the third development stage (156.56). According to the quality standards for forage legumes assigned by the Hay Marketing Task Force of the American Forage and Grassland Council (Rohweder et al., 1978) this study indicates that cutting red clover at any of the investigated stages of development will result in “premium” forage quality (RFV>151). RFV is not a direct measure of the nutritional content of forage, but it is important for estimating the value of the forage and is widely used as forage quality index in different legume crops (Tavlas et al., 2009; Asci, 2012; Tucak et al., 2013; Mariotti et al., 2020; Balazadeh et al., 2021).

Table 3. Forage quality of the red clover cultivar/population during different development stage (DS) in the first cut of the second year of growth 2018

Traits	CP Content (%)			ADF (%)		
Cultivar/Mean	VIVA	CD-3	Mean	VIVA	CD-3	Mean
I DS	19.10	20.30	19.70	26.57	26.27	26.41
II DS	17.66	18.47	18.07	27.00	27.30	27.15
III DS	15.56	15.67	15.62	31.30	32.10	31.70
Mean	17.44	18.14		28.29	28.55	
LSD 0.05ds	0.46			0.70		
LSD 0.05c	0.37			ns		
LSD 0.05ds × c	ns			ns		
Traits	NDF (%)			RFV		
Cultivar/Mean	VIVA	CD-3	Mean	VIVA	CD-3	Mean
I DS	30.36	31.50	30.93	209.15	202.27	205.71
II DS	33.07	33.20	33.13	190.92	189.57	190.25
III DS	37.47	38.87	38.17	160.19	152.92	156.56
Mean	33.63	34.52		186.75	181.58	
LSD 0.05ds	0.85			6.83		
LSD 0.05c	0.69			ns		
LSD 0.05ds × c	ns			ns		

Note: I DS of budding, II DS of beginning of flowering, III DS of full flowering.

One of the most common ways to determine concentration of available energy in the forage is by determining the total digestible nutrients. TDN content is related to the ADF content of the forage. As ADF increases there is a decline in TDN which means that animals are not able to utilize the nutrients that are present in the forage (Asci, 2012). Differences in terms of TDN content was determined among development stages of red clover (Table 4). The highest TDN was established in the first development stage (68.03%). TDN decreased with the aging of plants and the progression of maturity (63.86%).

The net energy of lactation (NEL) of forages is important for formulating the diets of dairy ruminants. For optimal milk production from lactating dairy cows, therefore, knowledge of the efficiency of energy utilization by ruminants and, consequently, of the nutritional quality of forage is indispensable (Johnson et al., 2003; Homolka et al., 2012). Significant differences between development stages were recorded for NEL, NEm and NEg (Table 4). All energy values were the highest in the first development stage (6.63 MJ kg^{-1} for NEL, 7.05 MJ kg^{-1} for NEm, 4.04 MJ kg^{-1} for NEg), and the lowest in the third development stage (6.03 MJ kg^{-1} for NEL, 6.54 MJ kg^{-1} for NEm, 3.53 MJ kg^{-1} for NEg).

Table 4. Nutritive value of the red clover cultivar/population during different development stage (DS) in the first cut of the second year of growth 2018

Traits	TDN (%)			NEL (MJ kg^{-1})		
Cultivar/Mean	VIVA	CD-3	Mean	VIVA	CD-3	Mean
I DS	67.91	68.15	68.03	6.61	6.65	6.63
II DS	67.57	67.33	67.45	6.56	6.53	6.54
III DS	64.17	63.54	63.86	6.08	5.99	6.03
Mean	66.55	66.34		6.42	6.39	
LSD 0.05ds	0.554			0.079		
LSD 0.05c	ns			ns		
LSD 0.05ds × c	ns			ns		
Traits	NEm (MJ kg^{-1})			NEg (MJ kg^{-1})		
Cultivar/Mean	VIVA	CD-3	Mean	VIVA	CD-3	Mean
I DS	7.04	7.07	7.05	4.02	4.05	4.04
II DS	6.99	6.97	6.98	3.98	3.95	3.97
III DS	6.58	6.51	6.54	3.57	3.49	3.53
Mean	6.87	6.85		3.85	3.83	
LSD 0.05ds	0.067			0.067		
LSD 0.05c	ns			ns		
LSD 0.05ds × c	ns			ns		

Note: I DS of budding, II DS of beginning of flowering, III DS of full flowering.

Correlation coefficients between investigated agronomic and forage quality traits are shown in Table 5. All researched agronomic traits (GMY, DMY, CPY, PH) had highly significant positive correlation relationships which ranged from 0.54 (CPY vs PH) to 0.96 (GMY vs DMY).

Also, all agronomic traits had a positive relation with the content of ADF and NDF, with the highest correlation coefficient found for ADF with DMY and PH (0.96 and 0.92). The most important forage quality trait CP content was positively correlated with RFV, TDN and energy values (NEL, NEm, NEg), but negatively correlated with ADF, NDF and all agronomic traits. RFV, TDN and energy values between themselves were in a strong positive correlation relationships and very significant negative relationship with agronomic traits, ADF and NDF. The correlation relations established in this work are consistent with research on the relationship between yield and yield revealed traits and forage quality properties in numerous legume crops (Tucak et al., 2013; Zhang et al., 2014; Marinova, 2017; Cacan et al., 2018; Seiam and Mohamed, 2020).

Table 5. Correlations coefficients between investigated agronomic and forage quality traits

Traits	GMY	DMY	CPY	PH	CP	ADF	NDF	RFV	TDN	NEL	NEm	NEg
GMY	-	0.96**	0.82**	0.88**	-0.84**	0.91**	0.90**	-0.90**	-0.91**	-0.91**	-0.91**	-0.91**
DMY		-	0.73**	0.89**	-0.87**	0.96**	0.95**	-0.95**	-0.96**	-0.96**	-0.96**	-0.96**
CPY			-	0.54*	-0.40	0.59*	0.58*	-0.58*	-0.59*	-0.60*	-0.59*	-0.60*
PH				-	-0.94**	0.92**	0.96**	-0.95**	-0.92**	-0.92**	-0.92**	-0.92**
CP					-	-0.92**	-0.91**	0.92**	0.92**	0.92**	0.92**	0.92**
ADF						-	-0.96**	-0.97**	-1.00**	-1.00**	-1.00**	-1.00**
NDF							-	-0.99**	-0.96**	-0.96**	-0.96**	-0.96**
RFV								-	0.97**	0.97**	0.97**	0.97**
TDN									-	1.00**	1.00**	1.00**
NEL										-	1.00**	1.00**
NEm											-	1.00**
NEg												-

**P < 0.01, *P < 0.05.

CONCLUSIONS

The stage of plant development at the time of cutting significantly affected yields and forage nutritive values of red clover compared to the choice of cultivar. Forage cut at early stage of development resulted in the lowest yields, plant height, content of ADF and NDF and the highest protein content and digestibility. During the plant maturation the yields increased and the forage quality rapidly decreased. The obtained results pointed that the most efficient cutting management of red clover is achieved, in order to optimize both yield and forage quality value, by cutting plants in the development stage between full budding and the beginning of flowering. The research of correlations between the investigated traits confirmed the existence of a negative relationship between yields and forage nutritive quality.

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