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Improvement of Forage Nutritive Quality of Alfalfa and Red Clover through Plant Breeding

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Abstract: Alfalfa and red clover are important perennial legumes for the production of high-quality fodder. The improvement of the forage quality of legumes is one of the strategic goals of breeding programs. Variation in quality traits (protein content (CP), neutral detergent fiber content (NDF), and acid detergent fiber content (ADF)) and relative feed value (RFV) among seven cultivars and 39 elite breeding populations of alfalfa and red clover was evaluated in the study. Significant differences were determined among the investigated cultivars/populations. Alfalfa populations L-8, 10, 12, 15, and 20 were characterized by a high CP content (up to 23.47%) and/or low NDF and ADF contents. The highest CP content in red clover was recorded in population CD-18 (21.89%), while the lowest NDF and ADF contents were determined in populations CD-19 and CD-4, respectively. High RFV was determined in alfalfa populations L-10, 12, 20, 15, 16, 8, 11, and 17 (prime fodder), and in red clover populations CD-4, 8, 16, 14, and 19 (premium-quality fodder). The identified superior alfalfa and red clover populations will be used to improve the nutritional value of forage crops in our breeding program, which will lead to the release of novel cultivars with improved forage quality.

Keywords: alfalfa; red clover; population; cultivar; crude protein; fiber; relative feed value; breeding

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1. Introduction

Alfalfa (*Medicago sativa* L.) and red clover (*Trifolium pratense* L.) are two of the most popular perennial forage legumes and rich sources of highly nutritional voluminous forage for livestock feed [1–4]. Alfalfa is a crop that provides a higher yield of proteins per unit area than any field crop, which makes it highly desirable for the production of hay and feed for domestic animals [5,6]. As a local protein resource, alfalfa can be used for various farm animals and thus serve in the production of milk and meat for human consumption [7]. Red clover provides high-value feed for grazing livestock and, compared to other legumes, has a lower rate of protein degradation during ensiling [3,8]. Both species are valuable crops due to their agronomic advantages and their numerous beneficial environmental effects in terms of preserving soil fertility and reducing groundwater nitrate pollution due to the reduction in inorganic fertilizer use, preservation of biodiversity, soil erosion protection, mitigation of climate change impacts, fossil fuel consumption, greenhouse gas emissions, etc. [9–15].

In view of an ongoing discussion about the negative impacts of imported protein-rich feed [16], regionally produced protein resources are generally favored when looking for environmentally friendly and GMO-free sources. Currently, only 56% of the crude protein (CP) used in European organic farming is of European origin [17].

Due to the low level and insufficient production of plant protein for both animal and human nutrition, in most EU countries, the improvement of the quality of legumes is one of the strategic goals of numerous breeding programs.

The quality traits of perennial legumes are of a very complex nature, and their expression is influenced by, besides genetic factors, environmental factors (especially drought through direct and indirect effects on plant morphology and physiology) and by applied management (cutting frequency, plant maturity stage at cutting time, and cutting height). Determination of the right moment for cutting is of the utmost importance for obtaining alfalfa and red clover forage with high nutritive value as well as meeting yield requirements [18–22]. The most important forage quality parameters include protein content, content of crude fiber, neutral (NDF) and acid detergent (ADF), fiber and lignin, digestibility of the forage, and leaf to stem ratio [23–26].

One of the main goals in alfalfa and red clover breeding for the improvement of forage quality is increasing protein content and fiber digestibility. Low amounts of non-digestible components, such as cellulose fibers (NDF and ADF) or lignin in alfalfa cultivars, have the potential to increase the feeding value of alfalfa for livestock by improving the forage fiber digestibility, and to increase harvest management flexibility [27,28].

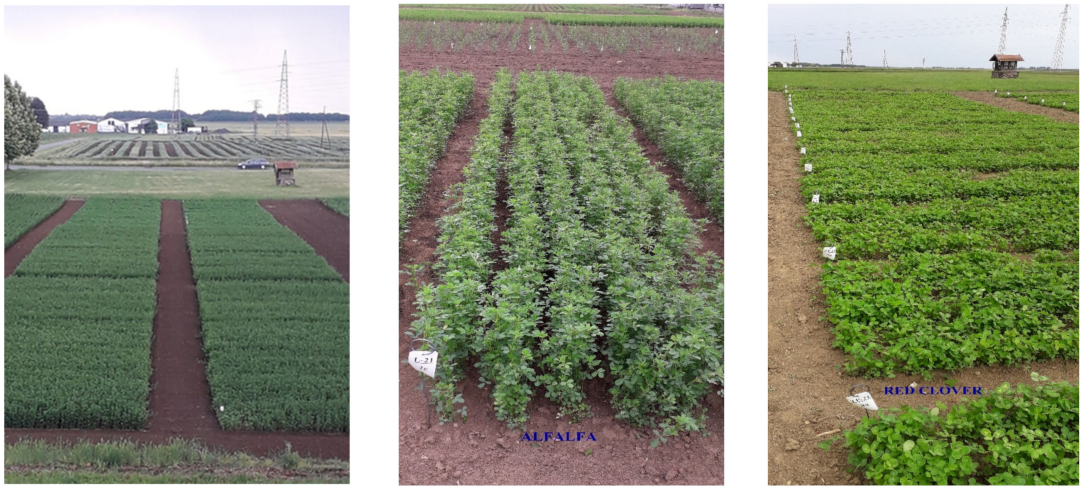
For the success and achievement of the abovementioned goals, the existence of wide genetic variability of major quality traits is necessary in the breeding process. Often, when improvements in agronomic traits are limited by low genetic variation, the most appropriate way to introduce new variation is to find new germplasm from the same gene pool. Plant introductions may be of more value in the early stages of breeding programs compared to using released forage cultivars as parents [29].

The aims of the study were to determine the variation of protein content and neutral and acid detergent fiber content in newly selected alfalfa and red clover populations/parental cultivars, to estimate relative feed value and to recognize desirable superior materials for the further improvement of quality properties in modern alfalfa cultivars.

2. Materials and Methods

The plant material included six older alfalfa cultivars (OS 70, Slavonka, Stela, Drava, Zdravka, and Vuka), one red clover cultivar (OS VIVA), and 39 elite breeding populations (alfalfa L1-20 and red clover CD1-19) created at the Department of Forage Crops Breeding and Genetics, Agricultural Institute Osijek, Croatia (45°32′ N, 18°44′ E, altitude 90 m). In order to set up a field experiment, the seed samples of alfalfa cultivars were obtained from the Croatian Gene Bank (CGB). Populations were developed after several cycles of phenotypic selection of individual plants with favorable agronomic traits produced using a large number of parental components (70% of divergent germplasm with broad genetic base and 30% of the abovementioned cultivars). Cultivars OS 70 and Vuka were used as parental components for the development of alfalfa populations L-2, 4, 5, 10, 12, and 19; cultivars Stela and Drava for L-1, 3, 7, 8, 9, 15, and 18; and cultivars Zdravka and Slavonka for L-6, 11, 13, 14, 16, 17, and 20. Red clover cultivar OS VIVA was used as a parental component for all red clover populations.

Samples of green mass (approximately 500 g) of each population/cultivar were taken in the second cut of the second growing season (2015) from three repetitions of an established field experiment, in which the forage yield and persistence of newly developed alfalfa and red clover populations were evaluated throughout several growing seasons (Figure 1). The experiment was conducted at the beginning of March 2014 using a randomized complete block (RCB) design with three repetitions. Plots were 1.4 × 6 m and consisted of 6 rows 20 cm apart. The sowing rate was 18 kg ha^{-1}. Soil chemical analysis was performed prior to sowing and revealed that the soil was well supplied with nutrients (pH in H_2O = 7.00; pH in 1n KCl = 6.41; AL-soluble P_2O_5 and K_2O = 39.70 and 37.70 mg 100 g^{-1}, respectively; humus content = 2.01%), so fertilizers were not applied. Herbicides were not applied in the trials.



(**a**) (**b**) (**c**)

Figure 1. (**a**) Experimental site at the Agricultural Institute Osijek, Croatia; (**b**) Alfalfa field trial; (**c**) Red clover field trial.

Samples were collected during the second cut, at the late bud to early flowering stage of alfalfa and at the beginning of the flowering to full bloom stage of red clover. Plants were cut at the height of 3 to 5 cm above the soil surface. All herbage samples were dried at 60 °C for 48 h, and plant tissues were ground with a Retsch (Type ZM1) ultracentrifugal mill with a 1 mm diameter sieve (Haan, Germany). The crude protein (CP) and neutral and acid detergent fiber (NDF and ADF) contents in dry matter (DM) were measured according to the standard protocol using FOSS Kjeltec 2300 and Velp Scientifica FIWE6 Raw Fiber Extractor. Feeding value parameters such as digestible dry matter (DDM), dry matter intake (DMI), and the relative feed value (RFV) were calculated according to the following equations adapted from common formulas for forages [30]:

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

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

RFV = (DDM% × DMI%)/1.29 (DDM—digestible dry matter, DMI—dry matter intake).

Standards for RFV as a criterion to grade hay were proposed by the Hay Marketing Task Force of the American Forage and Grassland Council [31], and are presented in Table 1. All collected data were processed by analyses of variance applying the least significant differences test using the general linear model procedure of SAS software 9.4 [32].

Table 1. Quality standards of legume, grass, and legume–grass mixture.

Quality Standard [a]	CP, %DM	ADF, %DM	NDF, %DM	RFV
Prime	>19	<31	<40	>151
Premium (1)	17–19	31–35	40–46	151–125
Good (2)	14–16	36–40	47–53	124–103
Fair (3)	11–13	41–42	54–60	102–87
Poor (4)	8–10	43–45	61–65	86–75
Reject (5)	<8	>45	>65	<75

Note [a]—standard assigned by Hay Marketing Task Force of the American Forage and Grassland Council; CP—crude protein; DM—dry matter; RFV—relative feed value; reference hay of 100 RFV contains 41% ADF (acid detergent fiber) and 53% NDF (neutral detergent fiber).

3. Results and Discussion

In grassland-based livestock farming, the perennial legumes alfalfa and red clover are the most common species used for feeding ruminant animals. Therefore, in forage crop

breeding programs, it is important that the newly developed cultivars are characterized, in addition to high forage yield, by good nutritional quality properties (high protein content, low fiber content, and good forage digestibility).

Analysis of variance demonstrated statistically significant differences in all of the quality traits studied, as well as in the relative feed value among investigated alfalfa and red clover populations/cultivars (Tables 2 and 3, Figure 2). In the different alfalfa population materials, CP content ranged from 21.25% (L-2) to 23.47% (L-8), while the average CP of all populations/cultivars was 22.26%. In addition to L-8, high CP was achieved in populations L-12, 10, and 15 (Table 2). These populations had a higher CP than all recognized cultivars, as well as a higher average value of all tested alfalfa materials (which was 1.2% higher for L-8), and represent valuable germplasm in breeding to improve quality properties. Population L-12 had the lowest NDF content (38.23%), and low NDF values were also recorded in L-10 and 20 populations. The highest NDF content was determined in population L-8 (44.64%). The lowest ADF content was observed in population L-10 (28.48%). Populations L-25, 26, and 12 also had lower ADF values, while population L-5 was found to have the highest ADF content (32.77%). NDF and ADF are both negatively associated with forage quality. As the NDF increases, animals are able to consume less forage; similarly, as ADF increases, digestible energy levels decrease. The detected levels of genetic variation for NDF and ADF contents among the studied populations in this research will contribute to an increase in the quality of selection materials created in the frame of our perennial forage crop breeding program.

Table 2. Forage quality traits of the investigated alfalfa populations/cultivars in the second cut of the second growing season at the late bud to early flowering stage.

Alfalfa	Quality Traits, %DM			Alfalfa	Quality Traits, %DM		
POP/CUL	CP	ADF	NDF	POP/CUL	CP	ADF	NDF
L1	21.55	32.52	41.82	L15	22.92	29.10	39.90
L2	21.25	31.66	41.13	L16	22.01	29.82	39.86
L3	22.76	31.96	41.56	L17	22.49	31.60	39.41
L4	22.06	31.37	40.09	L18	21.99	31.65	40.47
L5	21.98	32.77	39.19	L19	22.44	30.96	40.06
L6	21.73	31.61	41.38	L20	22.49	30.02	38.94
L7	22.87	30.96	39.41	OS 70	22.20	30.36	41.61
L8	23.47	29.32	44.64	Slavonka	21.64	30.84	41.53
L9	21.70	31.18	40.93	Stela	21.50	30.79	43.22
L10	23.11	28.48	38.74	Drava	22.61	31.69	44.37
L11	22.64	30.04	39.97	Zdravka	22.17	28.53	43.50
L12	23.15	28.97	38.23	Vuka	21.61	28.90	43.57
L13	22.68	30.39	40.93	Average	22.26	30.60	41.02
L14	21.76	30.20	42.06	LSD 0.05	0.35	0.61	0.57
				LSD 0.01	0.46	0.82	0.77

CP—crude protein; NDF—neutral detergent fiber; ADF—acid detergent fiber.

Avci et al. [1] evaluated the nutritive value of seven alfalfa cultivars with different fall dormancy rates over two consecutive years and determined in the second year of age significant differences among cultivars for the content of CP (from 17.9% to 19.7%; mean for year: 18.6%), NDF (from 44.5% to 49.4%; mean for year: 47.6%), and ADF (from 37.3% to 39.9%; mean for year: 39.1%). Sousa et al. [3] studied the nutritive value of eight alfalfa cultivars and one red clover cultivar over two years under less favorable growing conditions for alfalfa, and in the second year of cultivation, they found variation among alfalfa cultivars for CP (from 20.7% to 22.4%) and NDF (from 35.3% to 38.7%). Scholtz et al. [33] analyzed the chemical composition of 168 alfalfa hay samples and revealed a wide range of variation for CP (13.9–27.8%), NDF (28.9–65.9%), and ADF (21.3–47.3%). CP and NDF content values determined in the present study are similar to those reported by Sousa et al. [3], while the CP is higher, and NDF and ADF are lower than results obtained by Avci et al. [1]. A wider range of values observed in the literature for CP

and fiber fractions in alfalfa can be attributed to various factors, such as cultivar, climatic and agronomic management practices, and/or their interactions [34].

Table 3. Forage quality traits of the investigated red clover populations/cultivar in the second cut of the second growing season at the beginning of the flowering to full bloom stage.

Red Clover	Quality Traits, %DM			Red Clover	Quality Traits, %DM		
POP/CUL	CP	ADF	NDF	POP/CUL	CP	ADF	NDF
CD1	20.50	28.98	42.10	CD12	20.49	29.66	46.94
CD2	20.09	28.61	47.00	CD13	20.22	26.97	45.32
CD3	18.59	30.39	44.09	CD14	19.14	27.91	41.56
CD4	18.74	24.62	42.20	CD15	19.52	27.80	42.46
CD5	18.63	26.86	44.64	CD16	20.87	27.32	41.79
CD6	18.64	28.50	45.45	CD17	18.91	29.20	43.86
CD7	19.50	28.83	45.05	CD18	21.89	29.84	42.37
CD8	20.37	25.45	42.36	CD19	19.32	27.52	40.50
CD9	18.65	29.63	46.45	OS VIVA	19.58	27.54	44.69
CD10	19.56	28.79	42.34	Average	19.66	28.30	43.86
CD11	19.95	31.58	46.02	LSD 0.05	0.36	0.53	0.72
				LSD 0.01	0.48	0.71	0.96

CP—crude protein; NDF—neutral detergent fiber; ADF—acid detergent fiber.

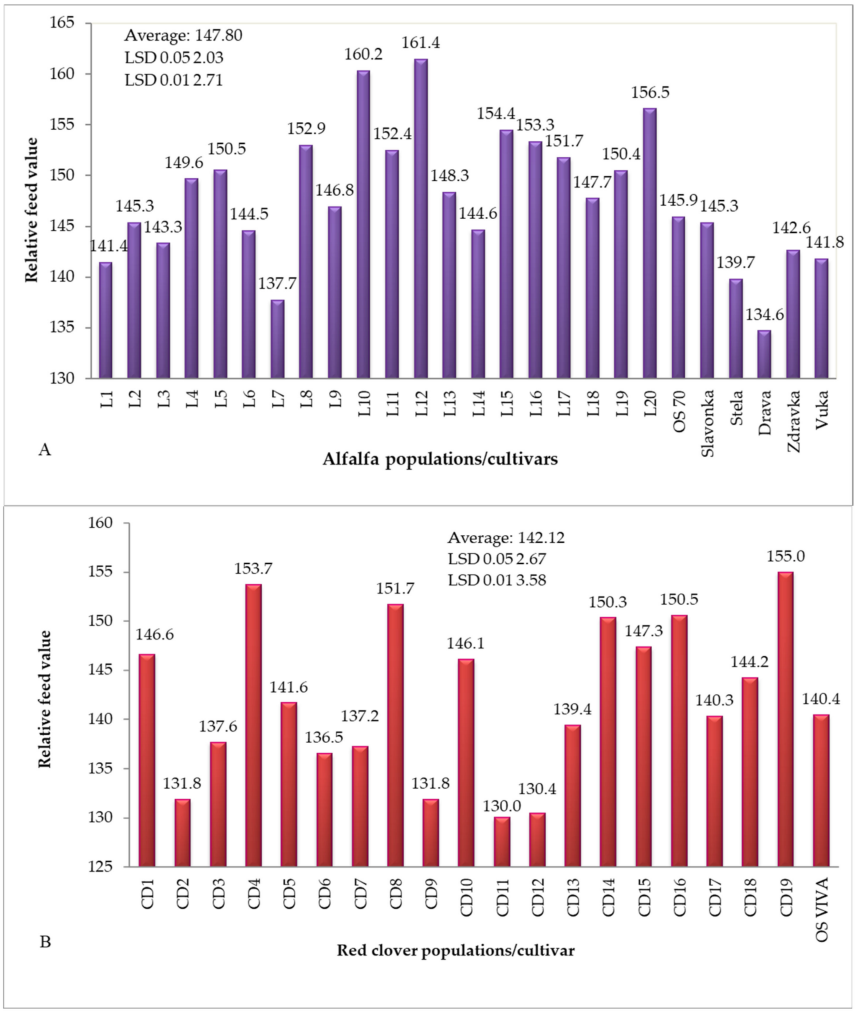


Figure 2. Relative feed value of the of the investigated alfalfa (**A**) and red clover (**B**) populations/cultivars.

Forage quality traits of the investigated red clover populations/cultivar are shown in Table 3. CP content ranged from 18.59% to 21.89%, while the average CP of all populations/cultivar was 19.66%. Population CD-18 was the highest and CD-3 had the lowest CP content. CD-18 achieved 2.31% greater CP content relative to the standard cultivar OS VIVA. Population CD-19 had the lowest NDF content (40.50%), while the highest content of NDF (47.00%) was recorded in CD-2. ADF content ranged from 24.62% to 31.58%, while the average ADF of all populations/cultivars was 28.30%. Population CD-4 had the lowest and CD-11 had the highest in ADF content. Favorable quality parameters (higher protein content and lower fiber content) were also recorded in populations CD-8 and 16. In this research, obtained CP contents are higher, while NDF and ADF contents are lower compared to the results obtained by Tucak et al. [35] when they evaluated variation in the yield and forage quality traits of domestic and foreign red clover materials. The values established in this paper for CP content are higher than those reported by Tavlas et al. [36] and Surmen et al. [37], while the NDF and ADF content values in the present study are similar to those reported by these authors. Differences in the values of the observed quality parameters obtained in this research and by the cited authors may be caused by genetic factors, but also depend on other factors, such as development stage, cutting height, growing year, environmental parameters, and their interactions.

The relative feed value in alfalfa populations/cultivars ranged from 134.64 (Drava) to 161.38 (L-12) (Figure 2A). According to the quality standards for forage legumes presented in Table 1, population L-12, as well as populations L-10, 20, 15, 16, 8, 11, and 17, is classified into the category of prime fodder. The relative feed value of red clover populations/cultivars ranged from 129.96 (CD-11) to 154.97 (CD-19) (Figure 2B). Except for CD-19, a high relative feed value was also determined in CD-4, 8, 16, and 14, and these populations are classified as premium-quality fodder. The relative feed value is widely used as a forage quality index in different legume crops [38–41].

Most newly developed populations of alfalfa and red clover had significantly better forage quality properties in relation to parent cultivars (L-4, 5, 10, 12, and 19 compared to OS 70 and Vuka; L-1, 3, 8, 9, 15, and 18 compared to Stela and Drava; L-11, 13, 16, 17, and 20 compared to Zdravka and Slavonka; CD-1, 4, 5, 8, 10, 14, 15, 16, 18, and 19 compared to OS VIVA). A larger number of superior populations were identified in both forage species, which confirms the necessity of introducing new genetic variability and divergent germplasm into the existing selection material. A number of genetic studies in forages have reported on the importance, potential, and advantages of the utilization of divergent genetic resources (crop wild relatives, landraces, and wild populations) in breeding strategies for the improvement of forage legumes cultivars, especially under the condition of global climate change, which is the most important challenge for the breeding of forage crops in the near future [42–45].

4. Conclusions

Significant variation was determined among the investigated alfalfa and red clover populations/cultivars in all of the quality traits studied, as well as in relative feed value. Most newly developed populations of alfalfa and red clover had significantly better forage quality properties in relation to parent cultivars. In both plant species, relative feed value ranged from 129.97 to 161.38 which, according to the quality standards for forage legumes (Force of the American forage and Grassland Council, Hay Marketing Task), is classified in the category of high-quality to excellent fodder. Several superior populations were identified in alfalfa (L- 8, 10, 12, 15, and 20) and red clover (CD-4, 8, 16, 18, and 19), and will be used to further improve the nutritional value of forage crops in our breeding program.

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