

CORRELATIONS OF MORPHO-AGRONOMIC TRAITS AND FORAGE
QUALITY PROPERTIES IN DIVERSE RED CLOVER
(*TRIFOLIUM PRATENSE* L.) COLLECTIONS

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Abstract: Red clover is an important perennial forage legume and a rich source of highly nutritional voluminous forage for livestock feed, which is continuously improved by plant breeding efforts that rely significantly on trait correlation studies. A two-year field trial was conducted at Rimski Šančevi, Novi Sad, Serbia. The aim of this research was to assess correlations of important morpho-agronomic traits and forage quality properties of 46 red clover accessions of diverse origins. The highest Spearman's correlation coefficient (0.97, $p < 0.01$) was found between the branch number and internode number per stem in the first experimental year. The green mass yield and the dry matter yield in both years had very high (0.95, $p < 0.01$) and high (0.86, $p < 0.01$) correlations, respectively. The stem height and internode number per stem had intermediate correlations in both years (0.68, $p < 0.01$, and 0.50, $p < 0.01$, respectively), and stem height exhibited an intermediate correlation with green mass yield (0.57, $p < 0.01$, and 0.62, $p < 0.01$, respectively) and dry matter yield (0.60, $p < 0.01$, and 0.56, $p < 0.01$, respectively) in both years. Thus, the indirect selection for higher plants with higher numbers of internodes per stem may contribute to accomplishing higher yield performance per plant. Although the branch number showed the highest positive correlation ($p < 0.01$) with internode number per stem and an intermediate positive correlation ($p < 0.01$) with stem height, this trait had a low correlation with green mass yield and dry matter yield and cannot be used as a criterion for indirect selection.

Key words: *Trifolium pratense* L., morphological traits, agronomic traits, indirect selection, forage quality.

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Introduction

A primary agricultural benefit of red clover (*Trifolium pratense* L.) is its ability to fix atmospheric nitrogen (N) via symbiosis with root nodule bacteria, Rhizobium, and to contribute available N to grasslands, improving soil fertility and increasing yield, both of subsequent crops with leys and companion grasses (Ciaran and Ratnieks, 2021). As animal feed, red clover has a higher N content than grass and can improve livestock performance via the superior nutritive value of grass plus clover forages versus grass alone (Fraser et al., 2004). It is widely sown for forage in Europe because of its high yield of digestible organic matter, metabolizable energy and protein content (Staniak, 2019). Usual tests for defining forage quality are dry matter (DM), pH, crude protein (CP), available protein, ammonia nitrogen (as % NH₃/TN), acid detergent fiber (ADF), neutral detergent fiber (NDF), lignin, and ash (Fulgueira et al., 2007).

Wild populations, as well as landraces and conventional cultivars of red clover, are naturally diploid ($2n = 2x = 14$), with gametophytic self-incompatibility, i.e. they need to be cross-fertilized before seed set. The modern tetraploid red clover cultivars ($2n = 4x = 28$) have been developed using autopolyploidy from diploid genotypes through chromosome doubling (Taylor and Quesenberry, 1996). The cultivated types of red clover had a similar level of genetic diversity, implying that modern red clover breeding programs did not negatively affect either genetic diversity or population structure (Osterman et al., 2021).

Genetic diversity is crucial to the breeders when improving agricultural plant species. The complexity of the red clover genome with high heterozygosity and heterogeneity has been an obstacle in genomic analyses (Li et al., 2019). Phenotypic distinctness in morpho-agronomic traits and forage quality properties within a population relies on genetic variation, but it is also influenced by environmental conditions (precipitation, temperatures) and by cutting frequency, plant maturity stage at cutting time, and cutting height (Swarup et al., 2021).

The favorable tendency of plant breeding encompasses the improvement of the multiple traits at the same time, however, it is difficult to achieve because of genetic correlations among different traits (Breseghello and Coelho, 2013). The correct assessment of genetic correlations requests large sample sizes and the existence of the genetic generic data, which may not always be available. Thus, phenotypic correlations are often supposed to reflect genotypic correlations (Sodini et al., 2018). The correlations are caused by pleiotropic genes, physical linkage of genes on the chromosome, or by the genetic structure of a population (Breseghello and Coelho, 2013). If two traits are favorably correlated, selection can simultaneously improve both. The indirect selection of chosen traits can lead to the improvement of the strongly positively correlated trait in the same direction

(Neyhart et al., 2019). On the other hand, unfavorable correlations are common and often cause problems for breeding.

The aim of this research was to assess and to analyze correlations of morpho-agronomic traits and forage quality properties of 46 red clover accessions tested in a two-year field trial and to show the performance of examined traits via frequency distributions. The obtained results can be used to consider the relationships of examined traits, especially the possibility for the indirect selection of traits that are strongly correlated with the main breeding goals, and for a simultaneous selection of multiple traits within the context of further red clover breeding.

Material and Methods

Plant material, field trial and experimental design

Plant material was selected from the red clover collection that belongs to the Institute of Field and Vegetable Crops, Novi Sad, Serbia. It was represented by 46 diploid and tetraploid red clover accessions, cultivars or populations originating from 17 different countries (Table 1).

The field trial was conducted at Rimski Šančevi (altitude 84 m, 45°20' N, 19°51' E), Novi Sad, Serbia, during the 2011 and 2012 growing seasons, and the sowing was performed at the beginning of April 2011. A field trial was arranged as a randomized complete block design with three replications (10 plants per repetition), with a row spacing of 80 × 80 cm (hill cropping), and a sowing depth of 2.5 cm.

The soil at Rimski Šančevi is a chernozem type, with extremely favorable pedological characteristics. The agro-chemical properties of the soil were analyzed in the Laboratory for Soil and Agroecology of the Institute of Field and Vegetable Crops, Novi Sad. Soil samples were slightly alkaline, with a medium content of CaCO₃, poor in humus, rich in total nitrogen, and with a high content of easily accessible phosphorus and potassium. No pesticides, chemical fertilizers, and irrigation were applied during the trial.

The location of Rimski Šančevi is characterized by a temperate-continental climate, with an average annual temperature of 11.3°C and an average precipitation of 631 mm. There was no significant difference in meteorological conditions between the two experimental years. During the vegetation period (from April to October), average monthly temperatures were higher than the long-term average. Maximum and minimum air temperatures were higher, while the total amount of precipitation was lower than the long-term average. The pronounced precipitation deficit was observed between June and September in both years. Relative humidity was generally lower than the long-term average during both years.

Table 1. The name, origin, type, and ploidy level of accessions from the red clover collection.

Genotype	Origin	Type	Ploidy level
89 E-0	Bulgaria	population	2n
91 E-44	Bulgaria	population	2n
91 E-63	Bulgaria	population	2n
Lutea	Germany	cultivar	2n
Amos	Denmark	cultivar	4n
Avala	Serbia	cultivar	2n
BGR1	Romania	population	2n
BGR2	Romania	population	2n
BGR3	Romania	population	2n
Bjorn	Sweden	cultivar	2n
Bolognino	Italy	population	2n
Bradlo	Slovakia	population	2n
Britta	Sweden	cultivar	2n
Čortanovci	Serbia	population	2n
Diana	Hungary	cultivar	2n
Dicar	France	cultivar	4n
Fertody	Hungary	cultivar	2n
Italia centrale	Italy	population	2n
Kora	Sweden	cultivar	2n
Krano	Denmark	cultivar	2n
Lemmon	Belgium	cultivar	2n
Lucrum	Germany	cultivar	2n
Marina	Serbia	cultivar	2n
Marino	Germany	cultivar	2n
Mercury	Belgium	cultivar	2n
NCPGRU2	Ukraine	population	2n
NCPGRU3	Ukraine	population	2n
NCPGRU4	Ukraine	population	2n
NCPGRU5	Ukraine	population	2n
Nemaro	Germany	cultivar	4n
Nessonas	Greece	cultivar	2n
Noe	France	cultivar	2n
NS-Mlava	Serbia	cultivar	2n
Quinekel	Chile	cultivar	2n
Renova	Switzerland	cultivar	2n
Rotra	Belgium	cultivar	4n
SA1	Australia	population	2n
SA3	Australia	population	2n
SA4	Australia	population	2n
Sofia52	Bulgaria	population	2n
Titus	Germany	cultivar	4n
Triton	Germany	cultivar	4n
Una	Serbia	cultivar	2n
Violeta	Bolivia	cultivar	2n
Violetta	Belgium	cultivar	2n
Vivi	Sweden	cultivar	4n

The evaluation of morpho-agronomic traits and forage quality properties

A detailed evaluation of the studied traits was carried out from the first cutting in July during both years. Each morpho-agronomic trait was determined by measuring 30 individual plants (10 plants per elementary plot) for each accession. Dry matter yield was determined after drying the plant samples at 105 °C to a constant weight.

In the first growing season, the following morphological traits were analyzed: internode number per stem (IN), branch number (BN), stem height (SH) (cm), stem thickness (ST) (cm), middle leaflet length (MLL) (mm), middle leaflet width (MLW) (mm), green mass yield (GMY) (g plant⁻¹) and dry matter yield (DMY) (g plant⁻¹).

In the second growing season, the following morpho-agronomic traits and forage quality properties were determined: internode number per stem, stem height, green mass yield, dry matter yield, crude protein content (CP) (AOAC, 1990), content of acid detergent fiber (ADF) (Van Soest, 1963), and content of neutral detergent fiber (NDF) (Van Soest and Wine, 1967). For the analysis of forage quality, plant samples were taken from the second cut of the second growing season, when about 25% of the flowers appeared (7 days after three heads of a plant had begun to flower). For each accession, three average bulk green mass samples were taken and dried at 60°C for 48h. Each bulk sample contained ten single plants.

Statistical analysis

The frequency distributions were determined for all investigated traits. The obtained mean values of agro-morphological traits and forage quality properties of red clover accessions were used to calculate the Spearman's correlation coefficient. The Spearman's coefficient was calculated according to the formula Eq. (1) (Zar, 1984):

$$r_s = 1 - 6 \sum d^2 / (n^3 - n) \quad (1)$$

where d is the difference between ranks for each x_i, y_i is a data pair, and n is the number of these pairs. This type of correlations represents a nonparametric criterion of the statistical strength for the relationship between two variables, and it is independent of the type of their distribution. In terms of intensity and strength, the values of the correlation coefficients (both Pearson's and Spearman's) can be classified into the following categories: very high (0.90–1.00), high (0.75–0.90), intermediate (0.5–0.75), low (0.30–0.50), and very low (0.00–0.30). The R software (R Core Team, 2017) was used to calculate Spearman's rank correlation coefficients.

Results and Discussion

The performance of studied red clover accessions

Frequency distributions of values of red clover morpho-agronomic traits in the first experimental year are shown in Figure 1.

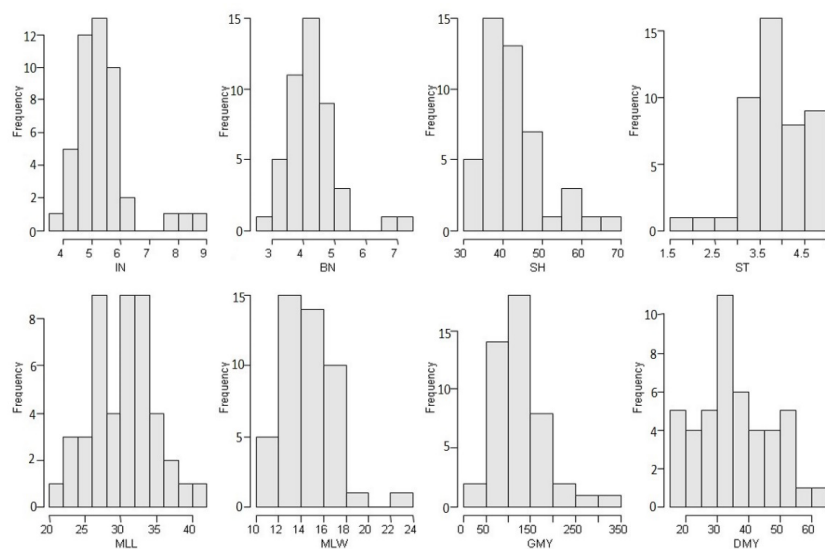


Figure 1. Frequency distributions of red clover traits in the first year. IN – internode number per stem, BN – branch number, SH – stem height (cm), ST – stem thickness (cm), MLL – middle leaflet length (mm), MLW – middle leaflet width (mm), GMY – green mass yield (g plant^{-1}), DMY – dry matter yield (g plant^{-1}).

The internode number per stem ranged from 3.87 to 8.53. The smallest branch number was 2.83, and the highest was 7.47. The variation for stem height was from 32.67 to 65.27 cm. The frequency distributions on the histogram for IN, BN, and SH were moved toward lower (below-average) values, and the most frequent classes were 5.0–5.5, 4.0–4.5, and 35–40 cm, respectively. The stem thickness ranged from 1.93 to 4.95 cm. The frequency distribution on the histogram for ST was moved toward higher (above-average) values, and the most frequent class was between 3.5 and 4.0 cm. The middle leaflet length varied from 21.97 to 40.10 mm, with the frequency distribution most resembling normal distribution with the three equal most frequent classes. The middle leaflet width showed variation from 10.60 to 23.40 mm. The green mass yield ranged from 40.33 to 319 g plant^{-1} . The frequency distributions on the histogram for MLW and GMY were moved toward lower (below-average) values, and the most frequent classes were 12–14 mm and

100–150 g plant⁻¹, respectively. The smallest value of dry matter yield was 16.77 g plant⁻¹, and the highest was 60.43 g plant⁻¹. The frequency distributions for DMY were uniform, with the exception of the most frequent class (30–35 g plant⁻¹) and the least frequent class (55–60 g plant⁻¹).

Frequency distributions of morpho-agronomic traits and forage quality parameters determined in the second year are shown in Figure 2.

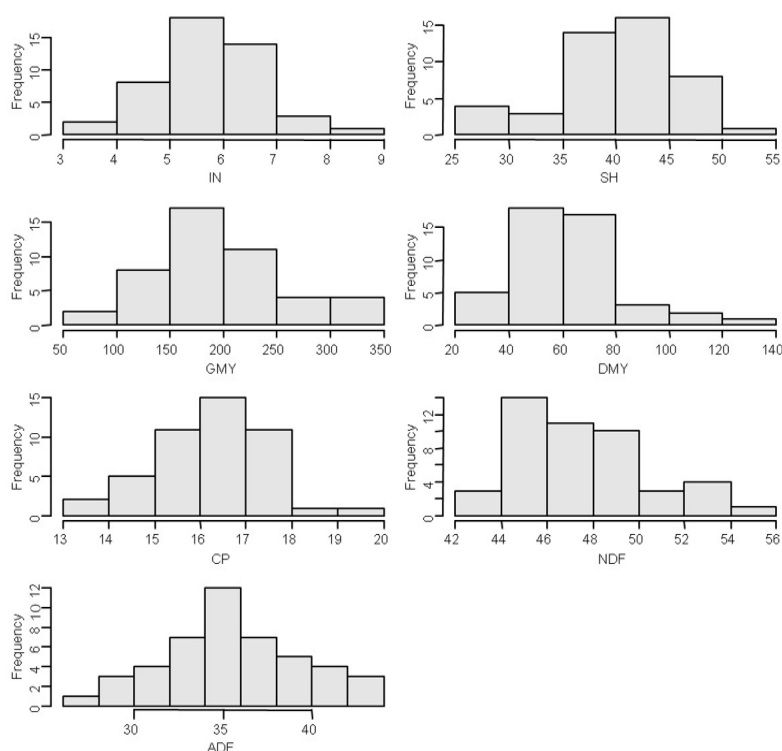


Figure 2. Frequency distributions of red clover traits in the second year. IN – internode number per stem, SH – stem height (cm), GMY – green mass yield (g plant⁻¹), DMY – dry matter yield (g plant⁻¹), CP – crude protein content (%), NDF – neutral detergent fiber content (%), ADF – acid detergent fiber content (%).

The internode number per stem was in the range of 3.33 to 8.73. The stem height of red clover accessions ranged from 26.77 to 50.23 cm. The lowest green mass yield and dry matter yield were 75.33 and 23.27 g plant⁻¹, respectively, and the highest values for GMY and DMY were 348.00 and 130.80 g plant⁻¹, respectively. The lowest value of the crude protein content was 13.73%, and the highest was 19.23%. The content of acid detergent fiber ranged from 27.78% to 42.60%. The content of neutral detergent fiber was in the interval from 43.08% to 55.54%. The frequency distributions for IN, GMY, CP, and ADF most resembled

normal distribution, and the most frequent classes were 5–6, 150–200 g plant⁻¹, 16–17%, and 34–36%, respectively. The frequency distributions for DMY and NDF were moved toward lower (below-average) values, and the most frequent classes were 40–60 g plant⁻¹ and 44–46%, respectively. The frequency distribution for SH was moved toward higher (above-average) values, and the most frequent class was between 40 and 45 cm.

Correlation analysis

The assessment of correlation coefficients is important for plant breeders from the aspect of the indirect selection of traits correlated with the main breeding objectives and for a simultaneous selection of multiple traits because a correlation between them can reduce the response to selection and expected genetic advance. The values of the Spearman's correlation coefficient of eight morpho-agronomic traits of red clover in the first year are shown in Table 2.

The highest positive significant correlation coefficient was found between the internode number per stem and the branch number (0.97, $p < 0.01$) (Table 2). Also, a very high, positive correlation (0.95, $p < 0.01$) was observed between the green mass yield and the dry matter yield. Middle leaflet length and middle leaflet width had a high correlation (0.77, $p < 0.01$). The correlation coefficients between internode number per stem and stem height (0.68, $p < 0.01$), branch number and stem height (0.64, $p < 0.01$), stem height and dry matter yield (0.60, $p < 0.01$), stem height and green mass yield (0.57, $p < 0.01$), were intermediate and positive.

Table 2. The Spearman's rank correlation coefficient for red clover morpho-agronomic traits in the first year.

	IN	BN	SH	ST	MLL	MLW	GMY	DMY
IN		0.97**	0.68**	0.37*	-0.05 ^{ns}	0.00 ^{ns}	0.41**	0.48**
BN			0.64**	0.38**	-0.06 ^{ns}	-0.04 ^{ns}	0.39**	0.47**
SH				0.36*	0.35*	0.42**	0.57**	0.60**
ST					0.41**	0.44**	0.32*	0.35*
MLL						0.77**	0.37*	0.43**
MLW							0.31*	0.34*
GMY								0.95**

ns – not significant; * – significant at the 0.05 probability level; ** – significant at the 0.01 probability level; IN – internode number per stem; BN – branch number; SH – stem height; ST – stem thickness; MLL – middle leaflet length; MLW – middle leaflet width; GMY – green mass yield; DMY – dry matter yield.

Low correlations were observed between the following morphological traits: internode number per stem and green mass yield (0.41, $p < 0.01$), internode number per stem and dry matter yield (0.48, $p < 0.01$), branch number and dry matter yield

(0.47, $p < 0.01$), middle leaflet length and dry matter yield (0.43, $p < 0.01$), stem thickness and middle leaflet length (0.41, $p < 0.01$), stem thickness and middle leaflet width (0.44, $p < 0.01$), stem height and middle leaflet width (0.42, $p < 0.01$).

Also, lower than previously, positive and highly statistically significant correlations were found between branch number and green mass yield (0.39, $p < 0.01$), and between branch number and stem thickness (0.38, $p < 0.01$). In the first growing season, a low correlation was observed between the following pairs of agro-morphological traits: stem thickness and green mass yield (0.32, $p < 0.05$), middle leaflet length and green mass yield (0.37, $p < 0.05$), middle leaflet width and green mass yield (0.31, $p < 0.05$), stem thickness and dry matter yield (0.35, $p < 0.05$), middle leaflet width and dry matter yield (0.34, $p < 0.05$), stem height and stem thickness (0.36, $p < 0.05$), stem height and middle leaflet length (0.35, $p < 0.05$), and internode number per stem and stem thickness (0.37, $p < 0.05$).

The values of Spearman's correlation coefficients among four morpho-agronomic traits and forage quality properties of red clover in the second experimental year are shown in Table 3.

Table 3. Spearman's correlation coefficients for red clover morpho-agronomic traits and forage quality properties in the second year.

	IN	SH	GMY	DMY	CP	NDF	ADF
IN		0.50**	0.27 ^{ns}	0.44**	-0.21 ^{ns}	-0.30*	-0.27 ^{ns}
SH			0.62**	0.56**	-0.26 ^{ns}	-0.12 ^{ns}	0.07 ^{ns}
GMY				0.86**	-0.08	-0.06 ^{ns}	-0.04 ^{ns}
DMY					-0.13	-0.11 ^{ns}	-0.12 ^{ns}
CP						-0.21 ^{ns}	-0.03 ^{ns}
NDF							0.43**

ns – not significant; * – significant at the 0.05 probability level; ** – significant at the 0.01 probability level; IN – internode number per stem; SH – stem height; GMY – green mass yield; DMY – dry matter yield; CP – crude protein content; ADF – acid detergent fiber content; NDF – neutral detergent fiber content.

The highest value of the correlation coefficient for the analyzed traits of red clover accessions in the second year was observed between green mass yield and dry matter yield (0.86, $p < 0.01$). The correlation coefficients between stem height and green mass yield (0.62, $p < 0.01$), stem height and dry matter yield (0.56, $p < 0.01$), and between internode number per stem and stem height (0.50, $p < 0.01$), were intermediate.

A low correlation was obtained for internode number per stem and dry matter yield (0.44, $p < 0.01$). The only significant correlation among forage quality properties, although low, was between the content of neutral detergent fiber and the content of acid detergent fiber (0.43, $p < 0.01$). The crude protein content was not significantly correlated either with morpho-agronomic traits or with other forage

quality properties. The only significant correlation between morpho-agronomic traits and forage quality properties, although very low, was shown between internode number per stem and the content of neutral detergent fiber (-0.30 , $p < 0.01$).

Many researchers (Vasiljević et al., 2006; Tucak et al., 2013; Hoekstra et al., 2018) identified significant relationships between green mass yield and dry matter yield and red clover stem height, suggesting that selection for a longer stem could lead to an increase of the biomass yield. Asci (2011) found the existence of a higher significant correlation between plant height and dry matter yield (0.87 , $p < 0.01$) than in our study and a smaller significant correlation between plant height and internode number per stem (0.30 , $p < 0.01$) than in our study. Tucak et al. (2013) found significant correlations for the following morpho-agronomic traits: GMY-DMY (0.93 , $p < 0.01$), GMY-PH (0.85 , $p < 0.01$), DMY-PH (0.81 , $p < 0.01$), GMY-NI (0.74 , $p < 0.01$), DMY-IN (0.65 , $p < 0.01$), PH-IN (0.79 , $p < 0.01$), GMY-MLW (0.72 , $p < 0.01$), GMY-MLL (0.79 , $p < 0.01$), DMY-MLW (0.54 , $p < 0.05$), DMY-MLL (0.62 , $p < 0.01$), PH-MLW (0.60 , $p < 0.01$), PH-MLL (0.84 , $p < 0.01$), MLL-MLW (0.72 , $p < 0.01$), which were similar to the results of our research, but with higher values, for all pairs of traits except for GMY-DMY and MLL-MLW, which had higher values in our research. These authors also reported a positive significant intermediate and high correlation of the internode number per stem and the middle leaflet width and the middle leaflet length, respectively, in contrast to our research, where insignificant correlations between these traits were observed. Unlike our research, which showed insignificant correlations between CP and ADF, and between CP and NDF, Tucak et al. (2013) reported significant negative correlations between CP-NDF (-0.73 , $p < 0.01$) and CP-ADF (-0.64 , $p < 0.01$), and positive between NDF-ADF (0.76 , $p < 0.01$). The most important forage quality parameters in red clover include protein content, the content of crude fiber, neutral detergent fiber content, acid detergent fiber content, the content of lignin, digestibility of the forage, and leaf to stem ratio (Tucak et al., 2021). Reiné et al. (2020) examined the nutritional quality of plant species in Pyrenean hay meadows of high diversity and, for red clover, determined negative associations between CP and ADF, CP and NDF, and positive between NDF and ADF.

Conclusion

Considering frequency distributions, the studied red clover collection had a considerable range of variability for the observed morpho-agronomic traits as well as for the forage quality properties. The stem height and internode number per stem had an intermediate, positive, and significant correlation, and stem height exhibited an intermediate significant correlation with green mass yield and dry matter yield. Thus, the indirect selection for higher plants with higher numbers of internodes per

stem may contribute to the selection toward accomplishing higher yield performance per plant. Although the branch number showed the highest, positive, significant correlation with internode number per stem and an intermediate, positive, and significant correlation with stem height, this trait had a low correlation with green mass yield and dry matter yield and cannot be used as a criterion for indirect selection. The only significant correlation between morpho-agronomic traits and forage quality properties, although very low and negative, was observed between internode number per stem and the content of neutral detergent fiber.

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KORELACIJE MORFOLOŠKO-AGRONOMSKIH OSOBINA I
POKAZATELJA KVALITETA KRME RAZNOLIKE KOLEKCIJE
CRVENE DETELINE (*TRIFOLIUM PRATENSE* L.)

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R e z i m e

Crvena detelina je važna višegodišnja krmna leguminoza koja predstavlja bogat izvor visoko nutritivne voluminozne krme za stočnu hranu, koja se kontinuirano poboljšava oplemenjivanjem. Dvogodišnji poljski ogled je bio postavljen na lokalitetu Rimski Šančevi u Novom Sadu, u Srbiji. Cij ovog istraživanja je bio da se utvrde korelacije unutar i između važnih morfološko-agronomskih osobina i pokazatelja kvaliteta krme za 46 varijeteta crvene deteline. Najviša visoko značajna vrednost za Spirmanov koeficijent korelacije (0,97, $p < 0,01$) utvrđena je između broja grana i broja internodija po stabljici u prvoj eksperimentalnoj godini. Prinos zelene mase i prinos suve materije su bili u značajnoj veoma jakoj (0,95, $p < 0,01$) korelaciji u prvoj godini, a jakoj (0,86, $p < 0,01$) korelaciji u drugoj godini istraživanja. Visina stabljike i broj internodija po stabljici su bili u umerenoj, pozitivnoj, značajnoj korelaciji u obe godine (0,68, $p < 0,05$, odnosno 0,50, $p < 0,05$). Visina stabljike je ostvarila umerenu, pozitivnu, značajnu korelaciju sa prinosom zelene mase (0,57, $p < 0,05$, odnosno 0,62, $p < 0,05$) i prinosom suve materije (0,60, $p < 0,05$, odnosno 0,56, $p < 0,05$) u obe godine. Indirektna selekcija na nivou biljke sa većim brojem internodija po stabljici može doprineti postizanju većeg prinosa po biljci. Iako je broj grana pokazao najveću, pozitivnu, značajnu korelaciju sa brojem internodija po stabljici i umerenu, pozitivnu značajnu korelaciju sa visinom biljke, broj grana je imao nisku korelaciju sa prinosom zelene mase i prinosom suve materije, i ne može služiti za indirektnu selekciju.

Cljučne reči: *Trifolium pratense* L., morfološke osobine, agronomske osobine, indirektna selekcija, kvalitet krme.

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