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GENOTYPIC AND ECOLOGICAL EFFECTS ON LEAFINESS OF RED CLOVER (*Trifolium pratense L.*)

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Abstract: The objective of this experiment was to: 1) examine the degree of influence of hereditary factors and conditions of growth (region of cultivation, season of growth and type of cultivation) on the phenotypic variance of leafiness in breeding populations of red clover; 2) quantify heritability and experimental variances in order to assess the opportunities for red clover breeding for improved leafiness. Five genotypes of red clover (four synthetic breeding populations and variety Sofia 52) were screened in the study. Among the factors studied as environmental variables, the season of growth had the strongest effect on the leafiness of red clover. In second vegetation of the life cycle of plants there was a significant additive genetic variance ($h_{ns}^2 = 0.31$) of the trait of leaf proportion in the fresh forage and the recurrent phenotypic selection for this criterion could be used in the breeding for leafiness in this species. It can be concluded from the results in this experiment that a higher degree of heritable genetic variation of leafiness must be searched for in connection with the variation of additional characteristics – thickness of stems, rates of formation and growth of stems, growth features related to regrowth (secondary growth) and age of sward (plants) and probably to persistence of genotypes.

Key words: red clover, leafiness, breeding, heritability

Introduction

Legumes are the major element of grassland ecosystems, to which the function to increasing forage quality is assigned. Leafiness is a particularly important morphological trait in forage legumes. Proportion of leaves in the forage positively correlates with main forage quality parameters – carbohydrates, protein and vitamins content and protein digestibility (*Brink and Fairbrother, 1992; Katic et al., 2005; Chourkova, 2011*).

Red clover leaves are an important ingredient of quality forage (*Vasiljevic et al., 2009*). Until the budding stage the protein content in plant is directly related to its content in the leaves and consequently to the degree of leafiness. The rate of decline in digestibility was lower in red clover leaves than stems *(Sanderson and Wedin, 1989*). Leaves in red clover are the morphological fraction, related also to another specific quality character of this species, namely content and activity of the polyphenol oxidase enzyme, which protects proteins and glycerol-based lipid in the rumen (*Sullivan et al., 2004; Michael et al., 2009; Parveen et al., 2010*). It was found that specific polyphenol oxidase activity was higher in red clover leaves compared to stems, while leaf age had no significant effect.

Although the parameters of proportion of leaves in the forage or leaf/stem ratio are main agronomic traits in variety testing of red clover (*Mihovski and Yancheva, 1998*), they are seldom used as an independent or main breeding criterion. On the one hand, the morphological types with great leafiness have usually lower productivity and persistence (*Goranova, 2002*). On the other hand, when using this species for haymaking, a part of leaf mass is lost to one extent or another in the process of harvesting and this makes the breeding improvement for this trait meaningless. However, in the aspect of using red clover for grazing, the breeding for great leafiness proves to be an important breeding aim and can be considered as breeding for productivity, since great leafiness of stems provides higher palatability and intake potential of forage (*Poli, 1998*). Ensiling is another main mode of using red clover, which also necessitates breeding for green forage quality, and consequently for leafiness.

Red clover is a polymorphic species. Proportion of leaves in total plant DM yield is primarily a function of morphological and biological type (*Goranova, 2002*), life cycle (*Brink and Fairbrother, 1992*) or level of ploidy (*Leto et al., 2004*), but is strongly influenced also by ecological conditions – temperatures and rainfall during the growth period (*Leto et al., 2004, Drobná, 2009*), intensity of sunshine, photoperiod, shading (*Rhykerd et al., 1959; Bowley et al., 1987; Taylor and Quesenberry, 1996*), diseases and insect pests.

The objective of this experiment was to: (1) examine the degree of influence of hereditary factors and conditions of growth on the phenotypic variance of leafiness in breeding populations of red clover; (2) quantify heritability and experimental variances in order to assess the opportunities for red clover breeding for improved leafiness.

Material and methods

Five genotypes of red clover, including four complex hybrid (synthetic) breeding populations, developed in one of our previous breeding works and variety Sofia 52, which was used as a standard variety, were screened in the study. The populations are a result of recurrent phenotypic breeding for specific adaptation to fore-mountain and mountain conditions of cultivation. In addition to productivity for haymaking use, they have been also consolidated according to different additional breeding criteria, namely: the material used for development of population A is late-flowering, for population B – with great leafiness and even seasonal distribution of yield, for S – with good persistence, for D – with high seed productivity.

The study was conducted under swards conditions at two locations (mountain and lowland region) during the following two growth cycles: 2005-2007 and 2008-2010, respectively (Table 1). In the first growth cycle the populations were cultivated alone and in binary mixtures with meadow timothy (*Phleum pretense L.*) variety Troyan, and during the second one – only as a continuous crop. The seeds of the experimental populations of red clover originated from two successive generations (Syn 1 and Syn 2).

Table 1. Geographic, soil and climatic data for the regions and the periods of the investigation.

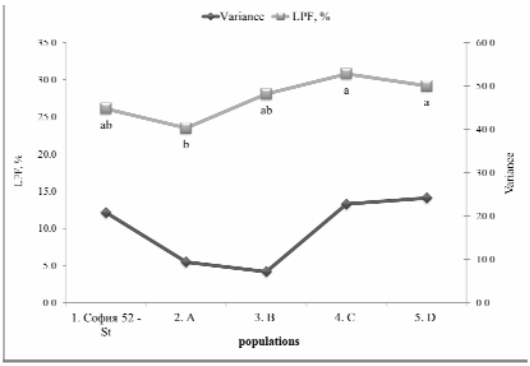
	Mountain region	**Lowland region**
Trial period	**2005-2007**	**2008-2010**
Location	**latitude – 42 ^{o} 88’ N** longitude – 24^{o} 72’ E **altitude - 384m**	**latitude – 43 ^{o} 24’ N** longitude – 25 ^{o} 32’ E **altitude - 144m**
Soil characteristics	**soil type – planosols. distric** soil pH - 5.4 in N_2O	**soil type – leached chernozem** soil pH - 7.1 in N_2O
Climatic data	rainfall amount during the growing season (April- October) average for the trial period - 1132 mm **mean annual temperature – 10.2^{o}S**	rainfall amount during the growing season (April- October) average for the trial period - 347 mm mean annual temperature – 12.8 ^{o}S

The experiments were carried out as randomised complete block design with 5 replications. The harvest plot area was 1 m^2. The sowing was conducted in early April for both growth cycles, at a sowing rate of 22 kg ha^{-1}. The leafiness was determined through the portion of leaf fraction in the mowed fresh biomass at haymaking ripeness of plants - at the stage of budding-early flowering. Through representative average samples of 1.5 kg in weight for each variant (or 0.3 kg from each replication) a morphological analysis of freshly cut biomass was performed. Weight percentage of leaves, stems and inflorescences in the fresh mass was determined. The number of obtained and hence analyzed cuts according to years was the following: 2006 – 3, 2007 – 1, 2009 – 2 and 2010 – 2. The populations were also characterized according to the following parameters: height of stems (HS, cm), variance of stem height (s^2), thickness of stems (TS, mm) and number of branches per stem (BS, nm). Biometric measurements were done on 30 stems from every genotype, 6 from each replication.

Data were analyzed with analysis of variance procedures and means comparison made with Least Significant Difference at 5% level of probability (LSD at $P<0.05$). The statistic program STATGRAPHICS PLUS was used. The studied sources of variation included two regions of cultivation (mountain or lowland region), growth season or cut (spring growth or summer regrowth) and two types of cultivation (alone or in mixtures). They were assessed in a factorial combination with the genotype factor. The setting of the trial did not allow an analysis of influence of the factor of "age of sward" (or age of plants), so the variance of leafiness was examined in separate dispersion complexes for second and third vegetation (for a two- or three-year sward, respectively). Degree of influence of the studied factors in the dispersion of leafiness was determined through correlation relationship (η^2). The coefficient of heritability, which was calculated as a share of the genetic variance from the observed phenotypic variance ($H^2_{bs}= \sigma_g^2/\sigma^2_{ph}$), was used as an indicator of existence of genotypic differences in the studied trait in the studied group of populations. The additive inheritance of leafiness was assessed through the coefficient of heritability in a narrow sense, which was calculated through regression (b) and correlation (r) of Syn 2 as compared to Syn 1 generation of populations ($h^2_{ns}=b/2r_{xy}$).

Results and Discussion

It was found that there was a significant effect of the conditions of growth (regions of cultivation - season of growth - type of cultivation combination), as well as of the genotype on the proportion of leaf mass in the forage during second vegetation, when the crop reached full development (Table 2). The highest values of leaf proportion in the forage were observed for populations S and D (Figure 1). In third vegetation, the studied factors were not a significant source of variability of leafiness. Then the breeding populations were inferior to the standard variety in leafiness with non-significant differences (Figure 2).



The means followed by same letters did not differ significantly, P=0.05

Figure 1. Leaf proportion in forage (LPF) mean for second vegetation, %

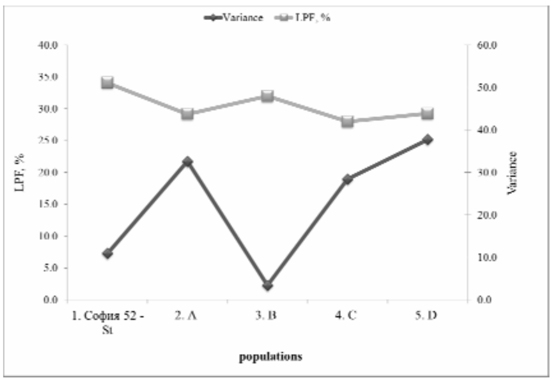


Figure 2. Leaf proportion in forage (LPF) mean for third vegetation, %

Table 2. Analysis of variance, significance and degree of factorial influences by leaf proportion in forage in red clover populations.

	Source of variation	df	MS	η^2
Second vegetation	E [a)]	5	35.9 *	0.29
	G	4	47.8 *	0.31
Third vegetation	E	1	5.7 NS	0.03
	G	4	47.8 NS	0.36
Second vegetation/ primary spring growth	R	1	7.41 NS	0.05
	G	4	32.90*	0.87
Second vegetation/ summer regrowth	R	1	72.04 P<0.10	0.29
	G	4	28.82 NS	0.47
Third vegetation/ primary spring growth	R	1	13.60 NS	0.03
	G	4	46.16 NS	0.46
Mountain region/ second vegetation	GS	1	11.03 NS	0.09
	G	4	21.91 P<0.10	0.73
Lowland region/ second vegetation	GS	1	82.6*	0.28
	G	4	47.5*	0.65
Lowland region/ third vegetation	GS	1	1.00 NS	0.01
	G	4	19.46 NS	0.51
Second vegetation/ primary spring growth	TC	1	0.48 NS	0.01
	G	4	26.61 NS	0.76
Second vegetation/ summer regrowth	TC	1	0.40 NS	0.01
	G	4	2.83 NS	0.14
Third vegetation/ primary spring growth	TC	1	0.65 NS	0.02
	G	4	60.87 NS	0.67

[a)] Environment (E) is a regions of cultivation (R) - growth season or cut (GS) - type of cultivation (TC) combination.
[*] Significant at the 0.05 probability levels

Region of cultivation and season of growth influenced the morphology of red clover by affecting the physiological processes of growth and development. Low soil moisture, increased temperatures and high sunshine intensity (characteristic of lowland conditions, as compared to mountain ones, or of the conditions of summer regrowth in comparison with the conditions of primary spring growth) limited the growth and as a consequence, they increased the portion of leaf fraction (*Leto et al., 2004; Drobná, 2009*). In this study at the same season of growth and equal age of sward (plants), the region of cultivation had a non-significant effect on the phenotypic variance of leafiness, in spite of significant differences in the values of growth ecological factors between mountain and lowland regions of study. Only in the summer regrowth during second vegetation there was a significant tendency (P<0.10, Table 2) to greater leafiness under the considerably drier conditions of the lowland region, by 4.7% on average for all populations. This result could be considered as an indicator of adaptive potential of the studied breeding populations.

Seasonal differences in ecological factors were greater in the lowland region, as compared to the mountain one. As a result, in lowland conditions the season of growth was a significant source of variability of the observed morphological trait ($P<0.05$, Table 2). In the summer cut of second vegetation, the portion of leaf mass, on average for all populations, was 5.7% greater than that in the spring regrowth, and in some genotypes (Sofia 52 and D) this increase reached to 9.1%.

Growth rate and morphological development of red clover were influenced by the processes of competition and synergism in mixed cultivation (*Pineiro and Harris, 1978; Guretzky et al., 2004*). According to the results, in mountain conditions the cultivation in mixture with meadow (common) timothy did not influence the leafiness of red clover (Table 2). In addition, there was no significant genotype reaction through leafiness to mixed cultivation. Timothy, as a component of the mixture, had slight regrowth, and in summer conditions it did not compete red clover practically.

The significant genotypic variance of leafiness observed in second vegetation was related to region of cultivation and season of growth ($P<0.05$, Table 2). Populations S and D reacted to dry conditions to the greatest extent through a great portion of the leaf fraction. According to our results, this was related not only to suppressed vegetative growth (smaller height of generative stems), but it was also a result of the variation of other characters, related to leaf proportion in the fresh forage – namely, morphology of stems, as well as rate of their formation and growth. The population D, at growth in dry conditions, formed considerably thinner and branched stems (Table 3), which increased the portion of leaf fraction. Growth of population S in the lowland region was characterized by uneven and continuous formation of generative stems (dispersion in the variation sequences for height of stems under mowing was the highest in this population, Table 3), which resulted also in high values of leaf proportion in the forage. This population has been bred for persistence and has heterogeneous biotype composition. The uneven rates of growth of stems were probably related also to greater biotype segregation in Syn 2 generation, which was cultivated in the lowland region. In population V, bred through recurrent phenotypic selection for the criterion of great leaf proportion in forage, there was a significantly lower variance of the trait according to regions, cuts and years (Figure 1, 2).

Table 3. Means of populations for height of stems (HS, cm), variance of stem height (s^2), thickness of stems (TS, mm) and number of branches per stem (BS, nm) at lowland region.

Populations	Second vegetation/ primary spring growth		Second vegetation/ summer regrowth		Third vegetation/ primary spring growth		Second vegetation/ primary spring growth	Second vegetation/ summer regrowth	Third vegetation/ primary spring growth	Second vegetation/ primary spring growth	Second vegetation/ summer regrowth	Third vegetation / primary spring growth
	HS cm	s^2	HS cm	s^2	HS cm	s^2	TS, mm			BS, nm		
Sofia52 St	58.0	84.4	40.0	68.1	64.1	34.3	4.7	4.8	4.8	2.3	3.7	2.6
A	45.9	63.8	40.8	70.7	68.9	138.6	4.7	4.4	4.3	2.8	3.2	3.3
B	51.4	39.5	31.4	69.6	62.6	72.7	4.4	4.4	4.5	2.2	3.4	3.0
C	53.6	84.3	33.6	118.6	56.5	491.6	4.2	3.7	4.0	2.0	2.8	2.9
D	58.9	58.2	44.7	52.3	65.1	80.3	4.4	3.2	3.8	1.7	4.2	3.2
Mean	53.6		38.1		63.4		4.5	4.1	4.3	2.2	3.5	3.0
$LSD_{0,05}$	2.7				5.1		0.4	0.6	0.5	0.4	0.8	0.7

As far as the rates of growth and development are a function of life cycle, the leafiness, being related to them, was influenced by the factor of age of sward to a great extent. In third vegetation there was a tendency to growth with finer and unequally tall generative stems, with more branches, due to natural thinning of swards (Table 3). This resulted in increased leaf proportion in the fresh forage, which had an average value of 37.0% for all populations with a respective value of 27.5% in second vegetation (Table 4). The setting of the trial did not allow an assessment of significance of this difference, but the age of sward (plants) was probably a factor having a considerable effect on the studied trait.

The observed significant genotypic influence in the total dispersion of leafiness exceeded the effects of the conditions of growth ($\eta^2_G > \eta^2_E$, Table 2). Broad sense heritability, as an indicator of interaction between leaf proportion in forage and genotype had a value of medium significance in second vegetation (H_{bs}^2 = 0.33, Table 4), and a low value – in the third one (H_{bs}^2 = 0.17). In addition, in second vegetation 31% of variation of leafiness was influenced by additive genetic causes (h_{ns}^2 = 0.31). In third vegetation there was no additive genetic variance (h_{ns}^2 = 0.03). According to the hereditary assessment of the additive effects, the trait had good response to breeding, but in the second year of life the plants. Also, the recurrent phenotypic selection for the criterion of leaf proportion in forage could be used with a good result with regard to phenotypic stability of the trait, which was confirmed by the results of population B bred for leafiness by the method specified.

Table 4. Variance components, broad sense (H^2_{bs}) and narrow sense (h^2_{ns}) heritability estimates for leaf proportion in fresh forage.

	Second vegetation	Third vegetation
Mean	27.5	37.0
Range	19.9-38.7	26.6-45.6
CV_{ph}	17%	14%
σ_e^2	12.1	26,8
σ_g^2	6.0	5.3
σ_{ph}^2	18.1	32.1
H_{bs}^2	0.33	0.17
h_{ns}^2	0.31	0.03

Conclusions

Among the factors studied as environmental variables, the season of growth had the strongest effect on the leafiness of red clover. In second vegetation of the life cycle of plants there was a significant additive genetic variance (h_{ns}^2 = 0.31) of the trait of leaf proportion in the fresh forage and the recurrent phenotypic selection for this criterion could be used in the breeding for leafiness in this species. It can be concluded from the results in this experiment that a higher degree of heritable genetic variation of leafiness must be searched for in connection with the variation of additional characteristics – thickness of stems, rates of formation and growth of stems, growth features related to regrowth (secondary growth) and age of sward (plants) and probably to persistence of genotypes.

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Uticaj genotipa i ekoloških faktora na olistalost crvene deteline (*Trifolium pratense* L.)

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Rezime

Cilj ovog eksperimenta bio je da se: 1) ispita stepen uticaja naslednih faktora i uslova rasta (region gajenja , sezone rasta i vrste uzgoja) na fenotipske varijanse olistalosti priplodnih populacija crvene deteline, 2) kvantifikuju naslednost i eksperimentalne varijanse u cilju procene mogućnosti za uzgoj crvene deteline za poboljšanu olistalost. Pet genotipova crvene deteline (četiri sintetičke populacije i sorta za priplod Sofia 52) su prikazani u studiji. Među faktorima koji su ispitivani kao promenljive iz životne sredine, sezona rasta je imala najjači uticaj na olistalost crvene deteline. U drugoj vegetaciji životnog ciklusa biljaka postoji značajna aditivna genetička varijansa (hns2=0.31) na osobinu udeo lista u svežoj krmi i rekurentna fenotipska selekcija za ovaj kriterijum može da se koristi u oplemenjivanju na olistalost ove vrste. Na osnovu rezultata u ovom eksperimentu može se zaključiti da viši stepen nasledne genetske varijacije olistalosti se mora tražiti u vezi sa variranjem dodatnih karakteristika - debljina stabljike, stopa formiranja i rasta stabljike, karakteristike rasta u vezi sa ponovnim porastom (sekundarni rast) i starost biljaka i verovatno sa perzistencijom genotipova .

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