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Comparative evaluation of diploid and tetraploid red clover genotypes in a flat area of Northern Bulgaria

Сравнителна оценка на ди- и тетраплоидна генплазма от червена детелина при равнинните условия на Северна България

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ABSTRACT

From the first to third growing season in the field test carried out at the Experimental Station of Soybean (Pavlikeni, Bulgaria) was observed 12 genotypes of red clover, including two Bulgarian diploid varieties Nika 11 (standard) and Sofia 52, four local diploid populations and next six foreign tetraploid varieties, i.e. Kvarta, Astur, Carbo, Elanus, Fregata and Larus. The goal of study was to determine the effect of ploidity level of the varieties as well as the effect of adaptive variability of local populations on fresh mass yield, dry mass yields and their persistence in an agro-ecological area with lower summer moisture assurance for red clover. The highest total dry mass yield was observed for varieties Sofia 52 (2n), Nika 11 (2n), Kvarta (4n) and Astur (4n). The productivity of tetraploids Kvarta and Astur strongly varies depending on the season and the year, as well as the age of sward. According to the obtained comparative production data for cuts and years, the tetraploid germplasm has a stronger adaptation to the limiting moisture. In summer drought, typical for the plains of Northern Bulgaria, tetraploids stop growing and do not compensate for their limited vegetative growth, despite the excellent moisture guarantee in the late summer, as well as in the spring the next growing season. Local wild-type germplasm of the species is characterized by a rapid development and lower yield of fresh and dry forage mass compared to the diploid standard when growing in conditions of optimum moisture but relatively high productivity in the dry conditions of the summer months. The differences in the genotypic productivity response under dry conditions reported in this experiment also suggest that the abiotic limit studied can be successfully responded by the genotype factor.

Keywords: diploid, drought, populations, red clover, tetraploid, variety, yield

РЕЗЮМЕ

От първа до трета вегетация в полски експеримент, изведен в Опитна станция по соята – Павликени, бяха наблюдавани 12 образеца червена детелина. В това число бяха включени двата български диплоидни сорта Ника 11 и София 52, четири местни диворастящи популации, както и следните шест тетраплоидни сорта: Kvarta, Astur, Carbo, Elanus, Fregata и Larus. Сорт Ника 11 е използван като стандарт. Целта на настоящото изследване е да се определи ефекта на нивото на плоидност на сортовете, както и ефекта на адаптивната изменчивост на местните популации върху фуражната продуктивност и дълготрайност на използване на тревостоите в агроекологичен район с по-ниска лятна влагоосигуреност за червената детелина. Най-висока обща продуктивност на суха фуражна маса е наблюдавана за българските диплоидни сортове София 52 и Ника 11, както и за тетраплоидите Кварта и Астур. Продуктивността на посочените тетраплоидни сортове много силно варира в зависимост от подрастта, сезона и възрастта на растенията. Според получените сравнителни данни за продуктивност по подрасти и години, тетраплоидната генплазма има по-силно адаптационно ограничение към водно-дефицитен стрес спрямо диплоидната. При лятно засушаване, типично за равнинните условия на Северна България, тетраплоидните сортове не подрастват и не компенсират ограничения си вегетативен растеж, въпреки отличната обезпеченост с влага през късното лято, както и през пролетта на следващата вегетация. Местната диворастяща генплазма от вида се характеризира с бързо развитие и по-нисък добив на свежа и суха фуражна маса в сравнение с диплоидния стандарт Ника 11 при отрастване/подрастване в условия на оптимална влажност. За две от популациите е отчетена сравнително висока продуктивност при лятно подрастване и те могат да бъдат използвани за отбор на растения с генотипна ценност в селекцията ни за специфична адаптация на културата към сухи условия. Отчетените в настоящия експеримент изразени разлики в генотипната реакция по продуктивност при условия на засушаване дават основание да се счита, че на проучваното абиотично ограничение може успешно да бъде отговорено чрез генотипния фактор.

Ключови думи: червена детелина, диплоиди, добив, популации, сортове, сухоустойчивост, тетраплоиди

INTRODUCTION

Red clover (*Trifolium pratense* L.) refers to leguminous forage plants with high productivity and quality. It is grown in pure swards or as a basic legume component in medium-term mixtures for combined use (Bozhanska, 2017). The most suitable for its development are moderately humid habitats with good soil fertility and low soil acidity. In the plain and hilly areas of Northern Bulgaria the soil conditions meet the requirements of the species but its productivity and longevity are limited by the summer water deficit. For economically justified growing of red clover in temporary grasses, annual rainfall amounts of at least 700-750 mm with sufficient summer rainfall are required (Ostrowski and Łabędzki, 2011; Verma and Ahmad, 2017). For Northern Bulgaria such humidity is only present in the fore-mountain and mountain areas, but there are limiting factors for the species - very low levels of phosphorus content in the soil and high acidity.

So far, a great number of red clover genotypes have been tested for the fore-mountainous conditions of Bulgaria with a breeding and introductory purpose, including modern European varieties with a practical significance and distribution (Goranova, 2002; Goranova et al., 2003; Mihovsky et al., 2003, 2005; Mihovsky and Naydenova, 2017). In these studies, the effect of ploidity level of the varieties as well as the effect of adaptive variability of local populations on fresh and dry mass yields, long-lasting grassland utilization, reproductive performance, and feed quality were identified. The present study is also aimed at assessing the effect of these factors, but in an agro-ecological area with lower summer moisture assurance for red clover.

MATERIALS AND METHODS

The experiment was carried out at the Experimental Station of Soybean (Pavlikeni, Bulgaria) during the period of 2014-2016. From the first to third growing season was observed 12 accessions/ genotypes of red clover, including two Bulgarian diploid varieties Nika 11 and Sofia 52, four local diploid populations with the numbers in the experiment LP1, LP2, LP3, LP4, respectively and next six foreign tetraploid varieties, i.e. Kvarta, Astur, Carbo, Elanus, Fregata and Larus (Switzerland originated). The Nika 11 variety was used as standard. The genotypes were sown in nursery inline, at a line length of 1.5 m with 0.5 m spacing between the rows and with a sowing rate of 1,000 seeds per row. The field trials were laid out with a randomized block design with two replications. Mineral fertilization and inoculation with *Rhizobium* bacteria were not applied. After sowing, the area was rolled. Herbicides and cover crops were not used as well.

In the sowing year, plants were harvesteded once – at the end of July (27 July 2014). In the second and third vegetation the plants were harvested in budding - beginning of flowering stage at a height of 40 mm, and in the second vegetation three cuts were harvested, and in the third vegetation - one cut, respectively. By cuts and genotypes the results for fresh mass yield (FMY) (kg/m) were recorded. The fresh mass of each row/replication is weighed immediately after cutting. For determination of dry matter content (%) and calculation of dry mass yield (DMY) (kg/m) samples of 200 g from each row/replication was taken and dried in laboratory conditions to constant weight at at 105 °C. Data have been recorded for formation of every harvested gut. The growth habit was determined immediately prior to the harvesting of the first cut in the three vegetations.

Agro-climatic and soil characteristics of the area of study

The physic-geographic conditions for the Pavlikeni area are typical of the hilly-plateaus of Northern Bulgaria. The altitude is 144 m. The average annual rainfall over a ten-year period covering the study period is 602.3 mm and the vegetation rainfall sum - 401.4 mm (Figure 1). The maximum monthly rainfall is in June.

Compared to the fore-mountainous conditions of Central North Bulgaria, annual and vegetative rainfall are respectively 241 and 200 mm less. The average monthly rainfall for the summer period is also significantly lower, 51.3 mm compared to 87.5 mm in the optimal conditions for growing the crop. During the years of the experiment, both, spring droughts (second vegetation) and strong July or August soil and atmospheric droughts were observed (Table 1).

The soil type is chernozem, 40-50 cm humus horizon, with good water holding capacity, 47% porosity and 15.2% quench humidity. The soil reaction is neutral (pH 7.05), the humus content is 3-4%, the stock with mobile phosphorus (5.3 mg/100 g), and nitrogen (0.23%) is medium and with potassium is good (48.4 mg/100 g).

Experimental data were analyzed with ANOVA procedures using Statgraphics Plus for Windows software package. Degree of influence of the studied factors in the dispersion of FMY and DMY was determined through correlation relationship (η^2). Differences between cultivars/populations and standard cultivar were evaluated by Fisher's Least Significant Difference (LSD) test at 0.05, 0.01 and 0.001 level of probability.

RESULTS

In the year of sowing, the diploid and tetraploid varieties studied form only leaf rosettes. Three out of the four tested local populations (LP1, LP2 and LP3) until mid-summer also form generative stems, showing a prostrate

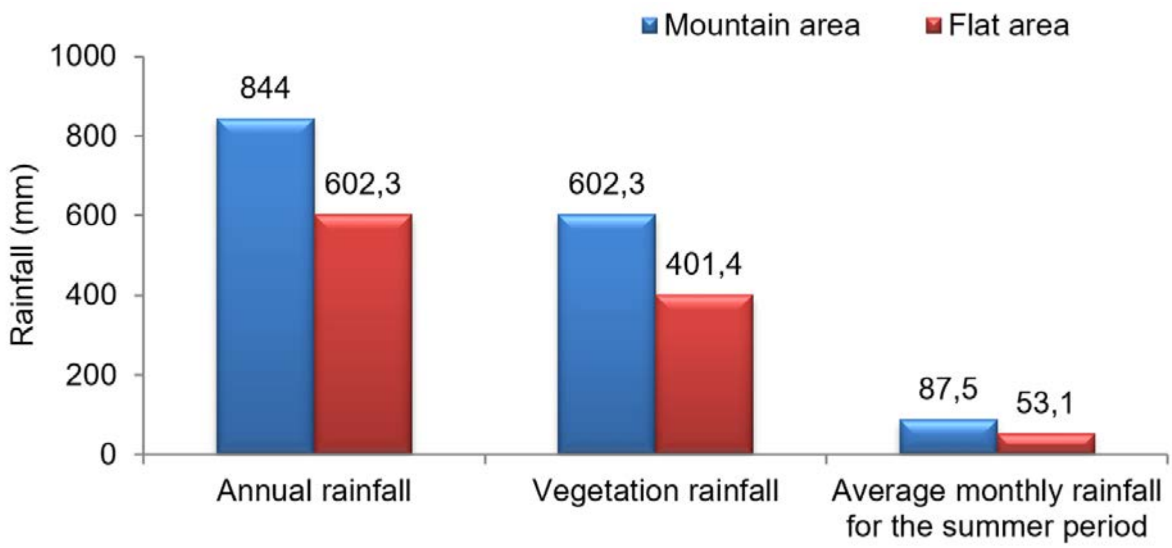

Figure 1. Characterization of the area of study relative to a mountainous area through the average rainfall for a ten-year period

Table 1. Meteorological data for the vegetation period (April to October) during the years of study

Months	First year		Second year		Third year	
	t	Rainfall	t	Rainfall	t	Rainfall
	°C	mm	°C	mm	°C	mm
April	13.2	67.8	12.2	39.7	16.2	51.4
May	17.8	93.3	19.8	66	16.6	105.9
June	21.6	90.6	21	86.4	23.7	67.6
July	23.4	105.8	25.6	20.4	25	25.4
August	24.3	11.6	24.8	72.3	23.8	66.9
September	18.3	115.2	19.9	46.8	19.7	37.1
October	12.4	94.1	11.6	37.4	11.4	38.4

growth habit. Genotypic differences in fresh and dry mass yield for the only harvested cut are significant (P<0.05, Table 2) and they due to the differences in the biological and morphological pattern of the genotypes.

In the second vegetation the periods in which the genotypes enter the harvest stage are as follows: 4-21 May for the first cut, 15-25 June for the second cut and 17-27 July for the third cut. The tetraploid specimens in July stopped to grow up. From the diploid Bulgarian varieties and local populations third cut was harvested. The period for formation of spring cut is 34 to 51 days, and for the summer cut 31 to 42 days, respectively (Table 2). The observed differences in growth and growth rates are not related to the level of ploidity. The local populations with the number LP 2 and LP 4, as well as the tetraploid variety Elanus were found the earliest. According to the analysis of the variance of fresh and dry mass yield, the genotypic influence is significant for the three cuts harvested in the second vegetation. In the first and second cuts, under the conditions of optimum humidity, the highest fresh and dry mass yield with an unproven difference to the Nika 11 standard was recorded for the diploid Bulgarian variety Sofia 52. The tetraploid varieties Kvarta, Astur, Carbo and Fregata fall into homogeneous group with the diploid standard and the tetraploid Elanus, as well as the studied local populations, yielded with significant differences (P<0.05).

In the harvesting of third cut under the conditions of drought, the highest yield from the diploid specimens was observed again for Sofia 52, as the Nika 11 gives him an insignificant difference. LP1 and LP2 populations are aligned with the standard by the fresh mass yield, but yield significantly to their dry mass due to the lower dry matter content.

In the third growing season one cut was harvested between 27 April and 12 May period. Spring growth of local populations is significantly faster than that of varieties (27 days versus 42 days). After the harvesting of the spring cut a significant plant dropout was observed, with line dilution being stronger in the tetraploid germplasm. The productiveness of tetraploids drops sharply in this vegetation and as a result the differences in favor of the diploid Bulgarian varieties were found the highest. The highest fresh and dry mass yield are obtained from the standard Nika 11, as the Sofia 52 and LP 2 yield with unproven differences.

The growth habit of the populations during spring growth is from prostrate to semi-prostrate, and in summer growth - semi-prostrate. The tetraploid varieties and the

Table 2. Fresh and dry mass yield, dry matter content and period of grow-up/growing for diploid and tetraploid red clover genotypes

Cultivars/ populations	First year			Second year												Third year			
	I cut			I cut				II cut				III cut				I cut			
	FMY (kg/m)	DM (%)	DMY (kg/m)	FMY (kg/m)	DM (%)	DMY (kg/m)	Terms (days)	FMY (kg/m)	DM (%)	DMY (kg/m)	Terms (days)	FMY (kg/m)	DM (%)	DMY (kg/m)	Terms (days)	FMY (kg/m)	DM (%)	DMY (kg/m)	Terms (days)
Nika 11 St	1.33	28.8	0.38	1.41	22.7	0.32	51	1.08	24.1	0.26	34	0.46	30.1	0.14	32	1.46	20.3	0.3	42
Sofia 52	1.57	25.5	0.4	1.68	25.6	0.43	51	1.3	22.6	0.29	34	0.56	32.8	0.18	32	1.37	21.9	0.3	42
LP 1	1.24	26.6	0.33	0.65	22.9	0.15	44	0.57	24.3	0.14	31	0.38	29.6	0.11	42	0.76	21.7	0.16	27
LP 2	1.07	27.4	0.29	0.75	23.5	0.18	34	0.68	28.6	0.2	41	0.36	26.6	0.09	42	1.23	20.9	0.26	27
LP 3	0.82	25.2	0.21	0.64	25.4	0.16	48	0.25	23.6	0.06	37	0.2	31.5	0.06	32	0.66	18.7	0.12	27
LP 4	0.37	25.9	0.1	1.09	22.2	0.24	34	0.75	26.2	0.2	41	0.32	31.8	0.1	42	0.43	22.5	0.1	27
Kvarta	1.62	26.3	0.43	1.55	23	0.36	51	1.08	20.0	0.22	34					0.82	18.4	0.15	42
Astur	1.34	25.3	0.34	1.49	20.4	0.3	44	1.21	22.2	0.27	41					0.7	20.1	0.14	42
Carbo	1	24.5	0.25	1.58	22.9	0.36	44	1.04	23.1	0.24	41					0.64	21.1	0.14	42
Elanus	0.9	22.2	0.2	0.97	19.9	0.19	34	0.59	22.4	0.13	41					0.45	18.8	0.09	42
Fregata	0.78	22.4	0.18	1.51	23.2	0.35	44	0.93	23.4	0.22	41					0.78	17.5	0.14	42
Larus	0.55	23.8	0.13	1.1	22.2	0.24	44	1.15	21.5	0.25	31					0.68	19.5	0.13	42
LSD0.05	0.3		0.08	0.27		0.06		0.22		0.04		0.11		0.01		0.29		0.06	
Mean for diploids	1.064	26.6	0.284	1.035	23.7	0.246	44	0.77	24.9	0.191	36	0.378	30.4	0.115	37	0.983	21	0.206	32
Mean for tetraploids	1.032	24.1	0.253	1.364	21.9	0.301	44	0.997	22.1	0.22	38					0.678	19.2	0.130	42

FMY – fresh mass yield; DM - dry matter content; DMY – dry mass yield; Terms – days for cut formation.

diploid standard variety Nika 11 are characterized by upright growth habit in all cuts and years. The growth habit of Sofia 52 is semi-erected. The forage biomass from the tetraploid varieties has higher humidity in all harvested cuts, with the difference between the mean values of the diploid and tetraploid indices remaining constant over seasons and years (Table 2).

Genotypic differences in total productivity of fresh and dry vegetative mass are significant (P<0.001) (Table 3).

Table 3. P-values from the analysis of variance of the total fresh and dry mass yield and degree of factorial influences η^2 (%)

	Genotype	Year, cut and age of sward
Fresh mass yield	***	**
Degree of factorial influences η^2 (%)	51.7	15.2
Dry mass yield	***	***
Degree of factorial influences η^2 (%)	24.3	11.6

**P<0,01; ***P<0.001.

The seasonal agro-climatic differences in cuts formation and plant age also have a significant effect on the variation in fresh and dry mass yields (P<0.001; P<0.01). The values of the main indicator η^2 for estimating this factorial influences on the forage productivity are 15.2 and 21.9%, respectively compared to 51.7 and 47.1% for the genotypic factorial influences (Table 3).

The highest total dry mass yield was observed for Sofia 52 variety (2n), with insignificant differences being inferior to the standard variety Nika 11 (2n) and tetraploid varieties Kvarta and Astur (Figure 2). It is important to note that the productivity of these tetraploids strongly varies depending on the season and the year, as well as the age of sward.

DISCUSSION

Red clover is a species with a low basic chromosome number (n = 7), which is a facilitator of autopolyploidization. The natural polyploidization of forage grasses is considered first as a mechanism for adaptation to unfavorable or new environmental conditions (Fawcett

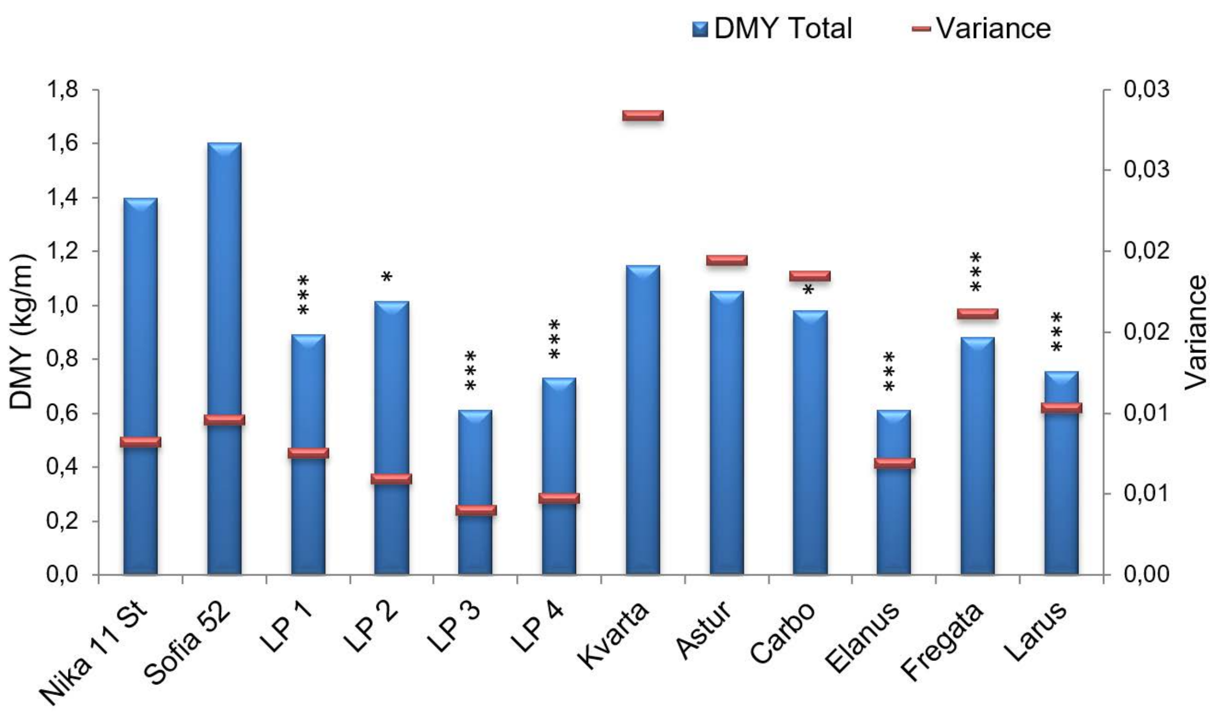

Figure 2. Total dry mass yield for the trial period (*,**,*** Significance of differences to the standard Nika 11 diploid variety: * Significance level is 0.05; ** Significance level is 0.01; *** Significance level is 0.001)

and Van de Peer, 2010). However, although red clover has a very wide distribution range, so far a natural polyploid form has been found only by Elci (1982) in a mountainous region of Eastern Turkey. It can be assumed that the adaptability strategy for this species is not related to polyploidization, but according to Goranova (2002) and Taylor (2008) - to a large biological and morphological polymorphism. For a very long time there have been well-established breeding techniques for inducing polyploid forms of red clover by treatment with colchicines, N_2O or by sexual polyploidization through unreduced gametes (Sattler et al., 2016). The tetraploid varieties registered in Europe do not give up on the practical distribution of the diploid. This is based on characteristics such as high forage productivity, increased stress resistance and persistence (Hejduk and Knot, 2010). However, according to a number of studies, the tetraploid varieties did not show any significant superiority over the diploid ones by dry mass yield (Liatukas and Bukauskaitė, 2012), by resistance to powdery mildew and sclerotia (McKenna et al., 2016), as well as by forage quality (Zuk-Golaszewska et al., 2010).

According to the Bulgarian studies conducted so far in the fore-mountain area, the tetraploid germplasm has no significant advantage over the diploid (Goranova et al., 2003; Mihovsky et al., 2005; Mihovsky and Naydenova, 2017). The tendency observed for these studies to lower total productivity of tetraploid varieties compared to diploid under the conditions of the present experiment, is very strong. According to the comparative production data for cuts and years, the tetraploid germplasm has a stronger adaptation to the limiting moisture. In this respect, the results of the analysis of the variance for fresh and dry mass yield can be interpreted as well. While under mountainous conditions it is predominantly due to the seasonal differences in climatic conditions and the age of the plants/swards (Mihovsky and Naydenova, 2017), under plain conditions, the genotypic factorial influences, including the level of ploidity is significantly stronger. In a comparative study of perennial leguminous forages in a flat Central European region with insufficient vegetation rainfall, Lang and Vejražka (2012) found the highest susceptibility to soil moisture deficiency during the secondary growth/summer growing season for red clover, more pronounced in tetraploid varieties than diploid. This is in agreement with the results obtained in the present study. In addition, the tetraploid germplasm in summer drought, typical for the plains of Northern Bulgaria, stops growing and does not compensate for its limited vegetative growth, despite the excellent moisture guarantee in the late summer, as well as in the spring the next growing season. The conclusion about reduced productivity and persistence of tetraploids on dry conditions or uneven distribution of rainfall during vegetation is also supported by Leto et al. (2004). Contrary to empirical evidence of high adaptive potential for water-deficient stress of tetraploid varieties but manifested as high forage yields in the first and second crop year, published by Tucak et al. (2016) and Novoselov et al. (2017).

There is little research on the mechanisms and genetic basis of drought tolerance in red clover, and in most of them the reaction to drought is traced in a controlled environment or concerns juvenile stages of the crop (Loucks, 2017). Naydenova and Georgiev (2013) found the increased tolerance to summer drought was associated with a higher degree of hairiness of the leaves with non-glandular trichomes. As mentioned, there is a large variation between modern varieties of red clover in tolerance to insufficient moisture. The differences in the genotypic productivity response under dry conditions reported in this experiment also suggest that the abiotic limit studied can be successfully responded by the genotype factor. Genetic center of origin and shaping of red clover refers to Southeast Europe and Asia Minor (Smith et al., 1995), i.e. the geographic centers of genetic diversity for this species include areas with more dry conditions, and there is, therefore, ecotype variability that has potential in the drought resistance breeding. According to Annicchiarico and Pagnotta (2012), the adaptive variability in wild red clover populations has a significant effect on performance by productivity and persistence in dry conditions. According to the results presented, wild-type germplasm of the species grown under the Mediterranean climate with long-term summer droughts, are equalized for the production of forage with the approved adapted varieties. Such results for productivity during the summer were observed for two of the populations (LP1 and LP2) included in this study. In another study, in a flat area for a local grazing red clover population significantly higher forage productivity was observed compared to the Sofia 52 variety under severe summer drought conditions (Naydenova et al., 2015). The population has already been used to select plants with genotypic value in the selection for specific crop adaptation to dry conditions. This approach has been successfully used by Claydon et al. (1993), which incorporate the high dry resistance of a wild-type germplasm of Mediterranean origin to a cultural, thereby creating a sustainable variety for pasture use in Australia.

Data showed the flowering in the year of sowing as well as the earliness by vegetation are not associated with lower longevity, as claimed by Choo (1984). Ford and Barrett (2011) and Annicchiarico and Pagnotta (2012) also found a positive relationship between earliness and persistence in dry conditions. The rapid development reported for local populations in the study can be considered as an adaptive biological feature that avoids drought as a stress factor. In addition, early flowering has been shown to facilitate invasiveness (Pyšek and Richardson, 2008) and such a characteristic of the local germplasm can be accepted as validated by natural breeding to provide self-sowing and self-maintenance of populations in the swards. Loucks et al. (2018) suggest that under conditions of water stress, red clover populations with different growth habit have different strategies of tolerance or survival. The observed change in the growth habit of local populations by cuts can be explained as a modifiable variability in response to changing climatic conditions in seasons.

The tetraploids Astur, Larus and Fregata varieties included in the study were created by colchicines treatment, the diploid varieties created on the basis of the Central European ecotype ("Mattenklee type"), i.e. they contain adaptive variability, transferred and recombined at tetraploid level (Boller et al., 2001, 2004). According to studies carried out in Central and Northern Europe, these genotypes are characterized by persistence, high and even in season's productivity, both alone and in mixed cultivation (Lehmann et al., 1998; Iepema et al., 2006; Hoekstra et al., 2018). This gives reason to believe that if polyploid forms based on wild-type germplasm, which has a natural specific adaptation to water deficiency, it can be considered that a high degree of dryness at the tetraploid level could also be achieved.

It is also important to note that, according to the results of the present study, the Bulgarian diploid varieties Nika 11 and Sofia 52, created by a recurring phenotypic breeding of family groups on long-term living and high summer productivity in the fore-mountain areas, show an adaptive potential for conditions of lower humidity than the one in which the breeding process was carried out.

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