NARDI FUNDULEA, ROMANIA ROMANIAN AGRICULTURAL RESEARCH, NO. 39, 2022
www.incda-fundulea.ro Print ISSN 1222-4227; Online ISSN 2067-5720

MORPHOLOGICAL AND CHEMICAL CHARACTERIZATION OF A COLLECTION OF MOUNTAIN CLOVER NATURAL POPULATIONS

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ABSTRACT

With climate change evident, the possibility opens up of introducing into production a species that, although not characterized by high yield, nevertheless offers some other benefits for both the environment and man. One of these species is mountain clover (*Trifolium montanum* L.), a species widespread around European in the past, but due to agricultural activities its habitat has become fragmented and areas of mountain clover impaired. In the present study, the collection of nine natural populations of mountain clover originating from different parts of the hilly-mountainous areas of Serbia was tested in field conditions. We analysed different morphological traits (green plant biomass, stem length, internode number, number of lateral branches, leaf length and leaf width), dry matter quality traits (content of crude proteins, crude fibre and crude fat) as well as secondary metabolites (total phenolic content, flavonoid contents and antioxidative activity). We collected morphological data and plant samples during 2011 and 2012. We performed descriptive statistics to provide basic information about variables in the dataset, then calculated Shannon-Weaver diversity index (H’) and performed two-way ANOVA and principal component analyses (PCA). Analysing the broad range of data collected during two years, we found considerable morphological and chemical diversity amongst the collection of mountain clovers from central Serbia. Mean coefficient of variation (CV) in the morphological dataset ranged from 18% (stem length) to 57.6% (plant biomass) in 2011 and from 16.5% (leaf length) to 70.6% (stem number) in 2012. Dry matter (DM) parameters displayed the lowest CV, ranging from 6.1% (crude proteins) to 14.8% (crude fat), indicating that these parameters were less discriminative within the study collection. Over all populations, average crude protein content was 19.5%, and average crude fibre content was 27.3%. Total phenolic contents (expressed as gallic acid equivalent, GAE) ranged from 49.8 to 89.7 mg GAE g^{-1} DM, and flavonoid contents (expressed as rutin, Ru, equivalent) ranged from 66.8 to 142 mg Ru g^{-1} DM. Average antioxidative activity expressed in terms of IC_{50} values ranged from 177 to 426 mg ml^{-1} of methanol extract.

Keywords: animal feed, field trial, genetic resources, legumes, *Trifolium montanum* L.

INTRODUCTION

Plant genetic resources (PGR) are the basis of food security and global energy (Nass et al., 2012) and its protection has become a major priority worldwide (Mellidou et al., 2020). PGR are the basis for creating highly productive genotypes that modern agriculture relies on. Sustainabile development of agriculture relies on use of natural resources (Malschi et al., 2010). Stable climatic conditions determine feed quantity and quality. But we cannot depend on climate stability. In the last decades, we are witnesses of dry periods, floods, late frosts, spreading of weeds and pests during production seasons. All those circumstances affect the breeder’s activities in two ways. The first is innovation and release of new crop cultivars (Brummer et al., 2011) and the second is research on marginal (neglected) species and exploring their potential for production.

One of those marginal species is mountain clover (*Trifolium montanum* L.), a legume, native for most European countries and Asia Minor. A map of its distribution can be found at The Euro + Med Plant Base website: http://euromed.luomus.fi/euromed_map.php?taxon=537851.

Mountain clover belongs to the fodder crop species, as a natural part of grasslands that are likely to play a part for future,

Received 7 June 2021; accepted 4 November 2021. First Online: November, 2021. DII 2067-5720 RAR 2022-25

sustainable agriculture. Those species provide nutritional and environmental benefits (Capstaff and Miller, 2018). In the past, grasslands have been transformed into arable land, for monoculture production. Grassland patches remained and their special isolation caused seed flow reduction (McConkey et al., 2012) and has disturbed the genetic structure of remaining species and their populations. Mountain clover has been studied as a model species that has suffered and declined in abundance due to habitat degradation and fragmentation (Schleuning et al., 2008). In addition, a particular problem with mountain clover is seed dispersal that is mainly bariochorus, and thus limited to short distances (Schleuning and Matthies, 2009). It is an element of semi-dry grasslands in the European Alps that have been increasingly fragmented over the last 150 years (Hahn et al., 2013). In contrast, larger grassland areas, such as at present on the Balkans, positively affect the existence of PGR. The Balkans is an area with less land consolidation and reduced industrialization that make it a very substantial source of fodder crop wild relatives, which are important in agriculture.

Our subject of interest was populations of mountain clover, collected from the central region of Serbia. As described by Zohary and Heller (1984) in their book "The genus *Trifolium*", *Trifolium montanum* is a clover which is perennial, pubescent or glabrous, with a woody stock, 15-80 cm. Stems many or few, erect or ascending, poorly branching above. Lower leaves with long petioles (up to 20 cm), upper ones short-petiole led to almost sessile, and stipules about 1 cm. Inflorescences capitate, 2-3 together, dense, many-flowered. Corolla white or yellowish, rarely pink.

This beautiful small clover is barely mentioned in the literature as animal feed. The quality and digestibility of dry matter of this species was studied by Ghanbari and Sahraei (2012) from an area of Iran. Up to now only one cultivar is in commercial production - *Guru* - created by the Research Institute for Fodder Crops, Ltd. Troubsko, Czech Republic. This cultivar can be grown in monoculture or in grass-legume mixtures.

The aim of the study reported here was to explore natural populations of mountain clover in field conditions and to assess its possibility for use as an animal feed. We also planned to collect, evaluate and characterise an important component of national genetic resources.

MATERIAL AND METHODS

Plant material

We used seed material of nine natural populations of mountain clover (Table 1). Seeds belong to the collection of the Institute for Forage Crops Kruševac, collected in hilly-mountainous regions of Serbia.

Table 1. Origin of plant material used in the field trail

Site name	Latitude	Longitude	Altitude, m a.s.l.
CEST	N 43°50'34''	E 21°40'36''	587.0
NINO	N 43°12'07''	E 20°17'03''	890.0
TARA1	N 43°53'26''	E 19°31'50''	984.0
TARA2	N 43°53'22''	E 19°32'28''	1043.0
GOCMP	N 43°30'37''	E 20°52'40''	677.0
GOCS1	N 43°32'37''	E 20°53'55''	911.0
GOCS2	N 43°32'57''	E 20°53'59''	914.0
KOPR	N 43°16'09''	E 20°52'22''	1068.0
KOPS	N 43°18'55''	E 20°51'15''	1465.0

Note. Site name indicates the mountain name and location: CEST - Čestobrodica; NINO - Ninaja, Osoje; TARA1 and TARA2 - Tara; GOCMP - Goč, Mitrovo Polje; GOCS1 - Goč, Stanišinci1; GOCS2 - Goč, Stanišinci2; KOPR - Kopaonik, Ravnište; KOPS - Kopaonik, Srebrnac.

Field trial

The experiment was conducted in the field of Institute for Forage Crops Kruševac (latitude 43°34'55", longitude 21°34'8") from autumn 2009 till summer 2012. The type of soil was degraded alluvial, characterized with pH in KCl of 6.57, organic matter content of 2.52%, available P_2O_5 content of 6.60 ppm and available K_2O of 24.05 ppm.

Seeds were scarified and germinated in plastic pots; plants were transferred to the field after developing 3-5 mature leaves, in late of September 2009. Each population was represented by sixty plants with 60 cm distance from row-to-row and 60 cm from plant-to-plant. Irrigation was applied once, after planting. During the following three years weeds were regularly removed with hand tools and motocultivator. Plant material was collected and analysed starting from 2011, because the yield of single plants during the period of the first cut of 2010 was too small to be worth analysing as most of plants were in the same condition as in autumn 2009.

Analysis of morphological traits and dry matter

To evaluate morphological diversity, seven traits were analysed: single plant biomass (PB) (cut at the soil surface), stem length (SL), number of internodes (IN), number of lateral branches (LB), leaf length (LL) and leaf width (LW). Measurements were performed in phase of first cut (from May to the June) during 2011 and 2012, when about 30% of flowers were open. This is recommended phase in agricultural practices because ratio of fodder quality and yield is the most favourable.

Analysis of dry matter quality and spectrophotometric analysis were performed in 2011, because we could not predict the amount of plant material in 2012, and number of plants that survived the winter.

Dry matter (DM) quality traits that are important for mountain clover breeding were analysed. After finishing the morphological analyses three samples of each population were prepared. Each sample consisted of 10-11 plants per population. All samples were dried in a plant drier for 48 h at 60°C. Dry material was ground in two mills, with sieve diameters of 2 mm and 1 mm. The contents of nutritionally important parameters (crude proteins, crude fibre, and crude fat) were analysed using the *Weende* system of analysis (AOAC, 1990). The content of crude protein was determined using Kjeldah method modified by Bremner. Crude fibre content was determined by refluxing in dilute base followed by dilute acid. Soxhlet method was applied to determine crude fat content.

Plant material preparation and spectrophotometric analysis

Plant material used in spectrophotometric analysis consisted of above ground parts of mountain clovers. From each population, three bulk samples were analysed. Each sample comprised parts of 3-5 single plants (cut during the beginning of flowering), air-dried in darkness, during 7 days at about 20°C. When drying was complete, material was ground, stored in dark glass jars until needed.

Ten gram of plant material was extracted with 200 ml of methanol; after 24 hours using Wathman No. 1 filter paper, the samples were filtered, and residues was re-extracted with equal volume of solvents. The process was repeated after two days. Combined supernatants were evaporated to dryness under vacuum at 40°C using Rotary evaporator. The obtained extracts were kept in sterile sample tubes and stored in a refrigerator at 4°C.

Total phenolic content was determined using Folin-Ciocalteu reagent (Singleton et al., 1999) and expressed in terms of gallic acid equivalent (mg GAE g^{-1} DM). Absorbance was measured with a spectrophotometer (ISKRA, MA 9523-SPEKOL 211) at wavelength (λmax) of 765 nm. Flavonoid contents were determined using the spectrophotometric method of Quettier et al. (2000) and expressed in terms of Rutin equivalent (mg Ru g^{-1} DM). The absorbance was measured at wavelength (λmax) of 415 nm. The ability of the plant extract to scavenge DPPH free radicals was assessed by the standard method (Tekao et al., 1994), adopted with suitable modifications (Kumarasamy et al., 2007).

Statistical analysis

Both data management and statistical analysis were conducted using statistical software *R* (R Core Team, https://www.R-project.org/). For each morphometric and chemical feature, descriptive statistics were calculated. Principal component analysis (PCA) was applied to identify the variability structure in the datasets. Two-way analysis of variance (ANOVA) was applied to morphometric data to examine differences in means among populations and years. The Shannon-Weaver diversity index (H′) was calculated according to Mellidou et al. (2020). According to Eticha et al. (2005) H′ was considered as high (H′ ≥ 0.60), low (0.10 ≤ H′ < 0.40) or intermediate (0.40 ≤ H′ ≤ 0.60).

RESULTS AND DISCUSSION

Morphometric characterization of the collection

For seven morphometric features, descriptive statistics of the average, minimum/maximum values, standard deviation, CV as well as Shannon-Weaver diversity index (H′) for samples in both 2011 and 2012 are presented in Table 2. Plant biomass (PB) and stem number (SN) gave the highest coefficient of variation (57.6-64.1% for PB, respectively, 57.0-70.6% for SN) (Table 2).

Considering the initial (starting) material, high population CVs are especially important, because they open the space for breeding activities. For IN and LB, coefficients of variation ranged from 20-30% during both experimental years. The lowest CVs were for SL (18.0%, 18.8%), LL (18.1%, 16.5%) and LW (21.1%, 17.0%), for 2011 and 2012, respectively (Table 2). High CVs during the two years and lack of the population × year interaction (Table 3), shows that morphological variability did not depend on the environmental conditions, but was a property of the analysed collection.

Table 2. Mean, standard deviation, CV%, standard error, minimum, maximum and H′ for 13 morphological and chemical features in a collection of nine populations of mountain clover

	Trait	Mean	SD	CV%	SE	Min	Max	H'
Morphology 2011	PB (g)	28.6	16.5	57.6	1.30	4	74	0.87
	SL (cm)	38.1	6.87	18.0	0.54	21	56	0.82
	SN	7.72	4.40	57.0	0.35	1	19	0.94
	IN	3.36	0.75	22.4	0.06	1	5	0.87
	LB	2.42	0.68	27.9	0.05	1	4	0.79
	LL (cm)	3.34	0.60	18.1	0.05	2.17	4.87	0.91
	LW (cm)	1.12	0.24	21.1	0.02	0.57	1.73	0.92
Morphology 2012	PB (g)	27.8	17.8	64.1	1.65	5	92	0.67
	SL (cm)	44.0	8.27	18.8	0.76	23	65	0.86
	SN	4.77	3.37	70.6	0.31	1	16	0.72
	IN	3.30	0.67	20.4	0.06	2	5	0.84
	LB	2.60	0.75	29.1	0.07	1	4	0.77
	LL (cm)	4.09	0.67	16.5	0.06	2.47	7.17	0.67
	LW (cm)	1.34	0.23	17.0	0.02	0.80	1.97	0.88
Chemical data	CP (%)	19.5	1.18	6.07	0.23	17.5	22.2	0.91
	CF (%)	27.3	2.05	7.51	0.39	23.3	32.0	0.91
	CFat (%)	2.21	0.33	14.8	0.06	1.74	2.89	0.94
2nd metabolites	AA	328	85.0	25.9	16.4	177	426	0.77
	TPhC	69.8	11.6	16.6	2.24	49.8	89.7	0.99
	TFC	102	19.6	19.2	3.77	66.8	142	0.77

PB (g) - single plant biomass, SL (cm) - stem length, SN - stem number, IN - internode number, LB - number of lateral branches, LL (cm) - leaf length, LW (cm) - leaf width, CP (%) - crude proteins, CF - crude fibre (%), CFat - crude fat (%), AA (mg ml^{-1}) - antioxidative activity, TPhC (mg GAE g^{-1} DM) - total phenolic content, TFC (mg Ru g^{-1} DM) - total flavonoids concentration.

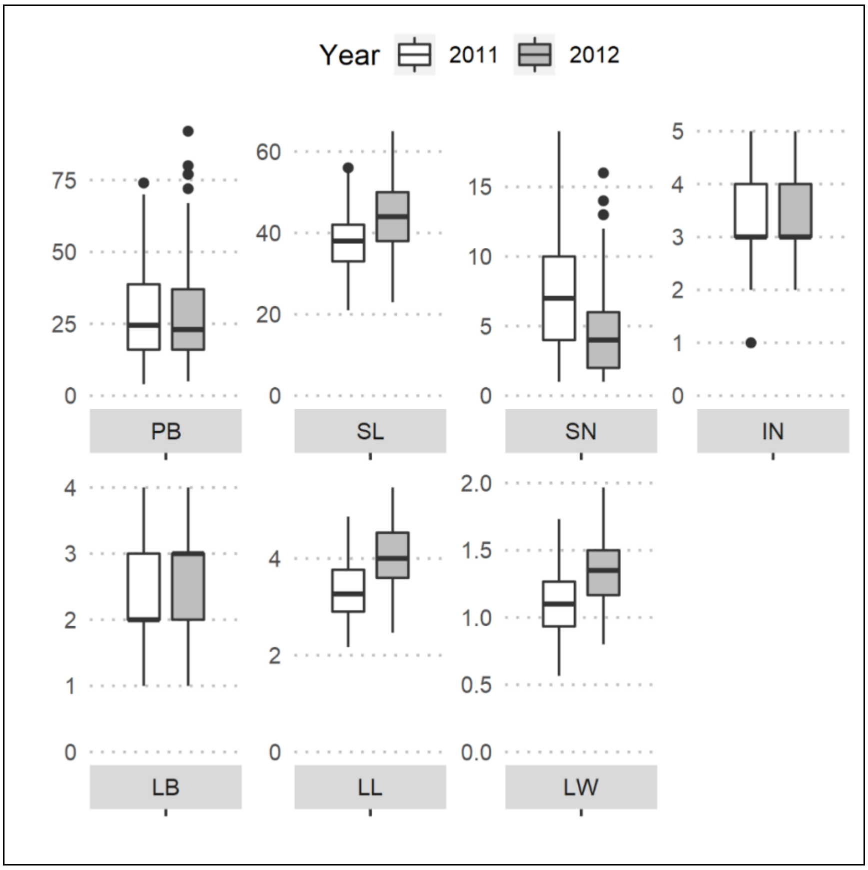
The Shannon-Weaver diversity index (H′) for morphometric traits ranged from 0.82 (SL) to 0.94 (SN) in 2011 and from 0.67 (PB, LL) till 0.88 (LW) in 2012 (Table 2). All traits during both years were characterized with H′ > 60, suggesting high morphological diversity of the mountain clover collection.

A two-way ANOVA was conducted to compare the effect of year and population on the morphological traits (Table 3). An analysis of variance showed that the effect of the year on SL, SN, LL and LW was significant. There was also a significant effect of population on PB, SL, LL and LW. The population x year interaction was significant only for leaf width.

Table 3. Two-way analysis of variance of mountain clover morphological traits

Factor	PB		SL		SN		IN		LB		LL		LW	
	F	p	F	p	F	p	F	p	F	p	F	p	F	p
Population	6.29	0.00	5.51	0.00	3.79	0.00	2.17	0.03	2.02	0.04	7.14	0.00	4.91	0.00
Year	0.19	0.66	45.8	0.00	39.9	0.00	0.58	0.44	4.73	0.03	118	0.00	73.4	0.00
Pop × Year	0.96	0.47	0.43	0.90	1.07	0.39	1.80	0.08	3.48	0.01	1.97	0.05	4.89	0.00

Results of morphometric analysis are additionally explained with box-plots (Figure 1). Box-plots show variation of SL, SN, LL and LW in different years. The values of LL, LW and SL were higher in 2012, but SN was lower in 2012, resulting similar plant biomasses for each population in both years. During this time, plant architecture changed towards elongation and increase of leaf area. We noticed a similar occurrence during seed collection, that plants of mountain clover growing up on the margins of grassland or on a country road (with plenty of space) were taller compared with plants from the middle of grassland. Lower levels of competition for light and soil minerals affect height of plant and increase the photosynthetic area.

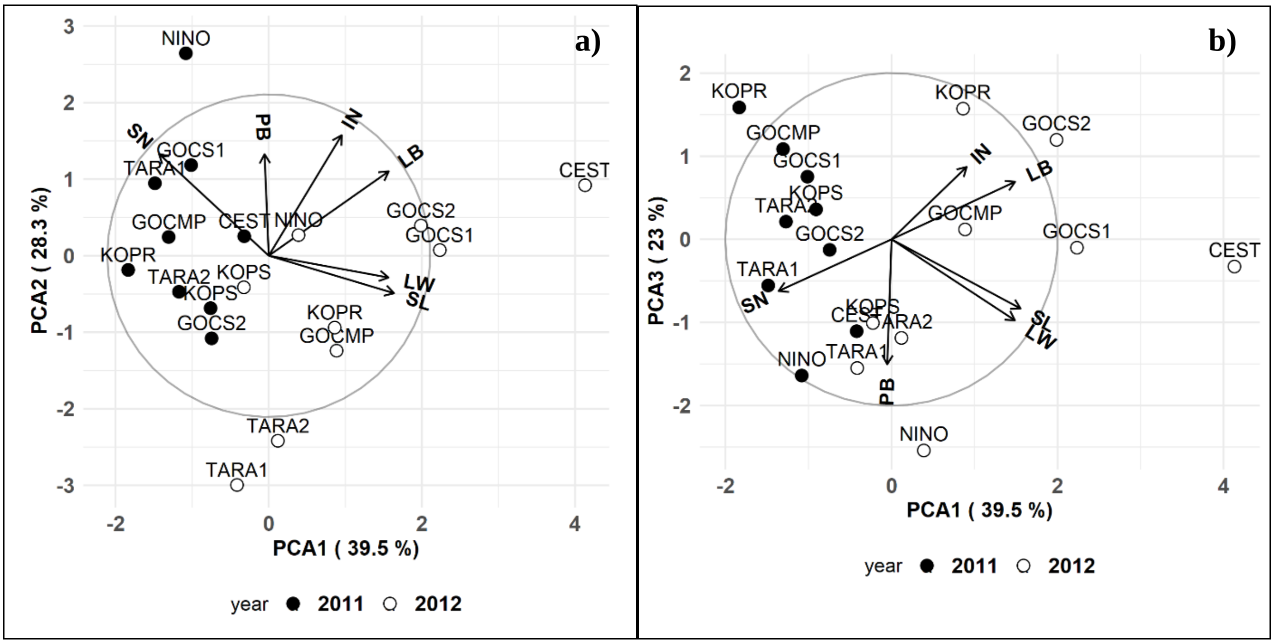


PB - single plant biomass, SL - stem length, SN - stem number, IN - internode number, LB - number of lateral branches, LL leaf length, LW - leaf width.

Figure 1. Box-plots of morphological traits in the 2011 and 2012 field trial

Principal Component Analysis (PCA) was applied to means of morphological traits for 2011 and 2012. Before analysis, we removed LL from the dataset because it was highly correlated with LW and SL. The first three principal components had eigenvalues larger than 1. The first component accounted for 39.5% of the total variance, the second for 28.3% and the third for 23.0% of total variance. Populations were clearly separated along the first axis (Figure 2). Populations from 2011 (located to the left in the figures) were characterized by large numbers of stems and smaller stem lengths and leaf size compared with the populations from 2012, shown to the right side in the figures. Plant biomass, number of internodes and stem number mostly contributed to PC2 (Figure 2a).

The trait responsible for separation along the third axis was plant biomass. Populations with lower biomass tended to be at the upper part of the graph (KOPR 2011, KOPR 2012), while populations with higher biomass (NINO 2011 and NINO 2012) were located at the bottom (Figure 2b).



PB - single plant biomass, SL - stem length, SN - stem number, IN - internode number, LB - number of lateral branches, LL leaf length, LW - leaf width.

Figure 2. Principal component analysis of morphological traits from 2011 and 2012
(a) PCA1 and PCA2, left, and (b) PCA1 and PCA3, right

Fodder quality

Crude protein contents ranged from 17.5% to 22.2% DM (Table 2). These protein contents are similar to those reported by Vasiljević et al. (2011) and Homolka et al. (2012) for red clover and alfalfa. Population TARA2 had the lowest crude protein content (18.5%), while KOPR had the highest (20.8%). Crude fibre contents ranged from 23.3% to 32.0% DM. Population GOCMP had the lowest crude fibre content (25.0%) while KOPS had the highest crude fibre content (29.9%). According to Dabkevičiene et al. (2016), high quality fodder should contain 14-17% proteins and 22-25% crude fibre. On this basis, we can conclude that fodder of mountain clover is of high nutritional quality as an animal feed. In our study, crude fat ranged from 1.74 to 2.89%, which was similar to the range measured in red clover by Vasiljević et al. (2011).

Coefficients of variation ranged from 6.1% (crude proteins) to 14.8% (crude fat), and those for CP, CF and CFat indicate that those traits are less informative for the analysed collection. The estimated Shannon-Weaver diversity index (H′) for all dry matter quality parameters was higher than 0.90 (Table 2), indicating a high level of polymorphism.

Secondary metabolites

Plant secondary metabolites are natural sources of biologically active compounds (Jurić et al., 2020) and play major roles in adaptation plants to their environment and in overcoming stress conditions (Akula and Ravishankar, 2011).

Antioxidant activities, expressed in terms of IC_{50} (μg ml^{-1}), ranged from 177 μg ml^{-1} NINO (890 m a.s.l.) to 426 μgml^{-1} GOCMP (677 m a.s.l.) (Table 4). As AA are expressed as IC_{50}, the lowest IC_{50}s indicate the strongest AA. The ability of mountain clover extracts to scavenge free radicals was weaker compared with Hungarian clover (Gođevac et al., 2008).

Total phenolic content ranged from 49.8 to 89.7 mg GAE g^{-1} DM, while flavonoids concentration ranged from 66.8 to 142 mg Ru g^{-1} DM (Table 4). The highest total phenolic contents were measured in populations GOCS1 (89.7 mg g^{-1}) and NINO (86 mg g^{-1}). The lowest TPhC was measured in samples from population GOCMP (61 mg g^{-1}) and KOPS (61.1 mg g^{-1}). Zlatić and Stanković (2017) found increases in the content of phenolic components as altitude increased from 285 m up to about 900 m. We found a similar trend here, though only until around 1000 m a.s.l., as TPhC contents in the material collected above 1000 m decreased.

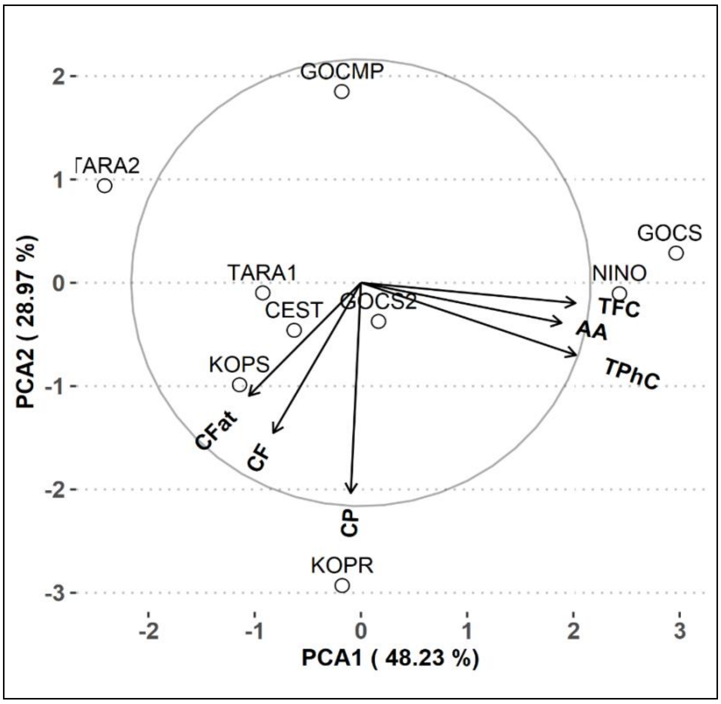
Table 4. Minimum and maximum of: antioxidative activities, total phenolic content and flavonoids concentration per population

	CEST		NINO		TARA1		TARA2		GOCMP		GOCS1		GOCS2		KOPR		KOPS	
	min	max	min	max	min	max	min	max	min	max	min	max	min	max	min	max	min	max
AA	343	343	177	180	377	378	400	402	425	426	220	221	313	313	277	279	420	421
TPhC	68.9	69.3	84.7	86	64.8	65.6	49.8	51.3	61	62.5	88.1	89.7	68	69.3	76.1	77.9	61.1	62.9
TFC	87.4	88.6	115	117	93.6	94.9	66.8	68.1	101	103	141	142	108	109	104	106	94.7	96

Populations with the highest flavonoid concentrations were GOCS1 (142 mg g^{-1}) and NINO (117 mg g^{-1}). The lowest flavonoid concentrations were measured in extracts of populations CEST (88.6 mgRu g^{-1}) and TARA2 (66.8 mgRu g^{-1}). Thus, no clear association between flavonoid concentrations and altitude was present, so the content of secondary metabolites may alternatively be related to the conditions of a certain habitat.

The CEST and TARA2 populations were collected on the edge of the forest and grew in partial shade, while the GOCS1 and NINO populations were collected in meadows where they were exposed to light throughout the growing season. A similar association was observed in the work of Alonso-Amelot et al. (2004) who found that higher light intensity in the habitat was accompanied by higher accumulation of secondary metabolites in certain organs. The highest CV was calculated for AA - 25.9%, while for total phenolic contents and flavonoid concentrations CVs were below 20%. H' was above 70% for AA, TFC and TPhC.

PCA was applied to population means of dry matter quality parameters and secondary metabolites (Figure 3). The first two principal components explained 77.2% of the variation in the data. The first principal component had positive association with TPC, AA and TPhC; the second principal component had strong negative association with CP, and negative associations with CF and CFat. Crude proteins were positively correlated with each of the parameters displayed in Figure 2. CF and CFat were negatively correlated with TPhC, TFC and AA. Populations that could be selected for further testing due to their positions on the chart are KOPS with a high content of crude proteins and NINO with the highest content of secondary metabolites.



CP - crude proteins, CF - crude fibre, CFat - crude fat, AA – antioxidative activity, TPhC - total phenolic content, TFC - total flavonoid concentrations.

Figure 3. The principal component analysis of biochemical traits for 2011 primary and secondary metabolites

Although seed material was collected in a relatively small area of central Serbia, populations showed significant differences in all three groups of analysed traits. The presence of this variability opens up the possibility of selection for improvement. As a perennial species with a deep tap root, mountain clover can stay on the same grassland for a long time and thus prolong the life of the grass-leg mixture. In this way, the costs of establishing the surface are avoided, weed development is prevented, grazing is provided, erosion is prevented, and one good quality cut is obtained per year.

CONCLUSIONS

Analysis of morphological properties, dry matter (DM) quality parameters, secondary metabolites and antioxidant activity showed the existence of variability of the tested collection of nine mountain clover populations from Serbia. The largest range of coefficient of variation was found for morphological traits: 18.0% (stem length) to 57.6% (plant biomass) in 2011 and 16.5% (leaf length) to 70.6% (stem number). Other groups of traits had lower CVs.

The crude protein contents in the most favourable phase of exploitation during the first cut ranged from 17.5% to 22.2% DM, for which the proportion of crude fibre was 23.3% to 32.0% DM.

Mean total phenolic content presented as gallic acid equivalent was 69.8 mg g^{-1}, the mean flavonoid content expressed as rutin equivalent was 102.0 mg g^{-1}. The mean antioxidant activity was relatively low at 328.59 µg ml^{-1} in terms of IC_{50}.

The results show that this collection of mountain clover populations can serve as starting material for creating a variety of mountain clover. However, due to the relatively low yield, it is not recommended to grow mountain clover in monoculture, but its longevity, good quality and high seed yield make it a desirable component of grass-legume mixtures for special purposes.

ACKNOWLEDGEMENTS

The research was financed by the Ministry of Education, Science and Technological Development, Republic of Serbia, project ref. number 451-03-9/2021-14/ 200217.

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