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Isoflavones of the red and Hungarian clover and possible impact on animal diet

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Abstract: The content of daidzein, genistein, formononetin, and biochanin A isoflavones was studied in natural populations of red and Hungarian clover, to estimate their impact on fodder quality and to determine directions in possible breeding programs. The study included 6 red clover (*Trifolium pratense*) and 6 Hungarian clover (*Trifolium pannonicum*) populations, collected in the central Balkans. The differences between the species and among the populations were analysed. The average content of total isoflavones was 1.393 mg g^{-1} and 0.487 mg g^{-1} of air dry matter in Hungarian clover, respectively. While the most prevailed isoflavone in red clover was biochanin A (46%), the Hungarian clover populations were rich in genistein (43%). The red clover leaves accumulated the highest content of isoflavones. The Hungarian clover flowers and leaves had an equal amount of isoflavones. The obtained values of the total isoflavones could not affect the overall nutrient quality and therefore, researched natural populations of two clover species could be considered for further breeding programs.

Keywords: phytoestrogens; *Trifolium pratense*; *Trifolium pannonicum*; daidzein; genistein; formononetin

Flavonoids are a widespread class of phenolic metabolites in plants. They are divided into several groups, including isoflavones which are common in the *Fabaceae* family (Reynaud et al. 2005).

The beginning of research on isoflavones is related to their negative effect on animal health – oestrous cycle disorders (Francis et al. 1967). Although high concentrations of isoflavones are thought to be harmful, lower amounts of phytoestrogenic compounds may be desirable (Sazdanić et al. 2018) due to benefits in the nutritional-physiological and health-promoting effects known for polyphenols (Kroyer 2004). Some studies showed a positive effect of isoflavones on animal weight, the fermentation process, and acidity reduction in the rumen (Jiang et al. 2007; Balcells et al. 2012). Isoflavones exhibit impressive anti-inflammatory effects in both humans and animals (Yu et al. 2016). The researchers have not yet shown precise doses of isoflavone intake that could be harmful to animals. Future experiments should address the precise dose-response effects of isoflavones on animal health and welfare.

This group of plant secondary metabolites is widely present in legumes. However, their total content and the presence of individual isoflavone components greatly vary among species, as well as among populations of a single species (Dabkevičienė et al. 2012). Red clover (*Trifolium pratense*) is known among the most important forage legumes because of its high nutrition value and high adaptability regarding occurrence

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on different land types and environmental conditions (Řepková and Nedělník 2014). Today, this species is an unavoidable part of a good-quality animal diet. Due to the relatively high content of isoflavones in red clover (Řepková and Nedělník 2014), special attention should be paid to the use of different genotypes in animal diet and related effects on the overall animal health (Mueller-Harvey 2013).

Hungarian clover (*Trifolium pannonicum*) has the longest exploitation span, and livestock prefers to consume it because of its good taste, despite the relatively thick hairy cover on its leaves and stems (Szabo 1987).

Hungarian clover provides balanced yield during a period of about seven years with two harvests each year. The species is suitable for growing in dry conditions and scharacterised by some higher content of mineral elements than the red clover (Pelikán et al. 2016).

Natural populations of red clover and Hungarian clover were collected in the central Balkans, i.e. the territory of Serbia, scharacterised by very variable environmental conditions, mainly climate and soil types. Wild populations represent the genetic resources serving as a valuable material for the development of modern stress tolerant and/or high-yielding varieties (Radinović et al. 2018).

This study aim was to explore the variability in the content of total isoflavones and their four individual isoflavone compounds present in different plant parts (leaf, stem, flowers, and whole aerial parts) of natural populations of red and Hungarian clover, regarding known isoflavones impact on animal health. The genotypes which will be included in breeding programs for improved animal fodder development should satisfy the needs of safe and good nutrient quality for health promoting animal diet.

MATERIAL AND METHODS

Plant material and trial conditions. The experiment was conducted on the property of the Institute for Forage Crops (Globoder, Kruševac, Republic of Serbia; altitude 150 m, 43°34'55" latitude, 21°34'8" longitude). Seeds of 12 different populations, six each of the red and the Hungarian clover were collected from different parts of the country (Table 1).

The collected seeds were scarified with water sand to break the dormancy and then the material was germinated in containers. When the plants reached the stage of 3–4 mature leaves, they were transferred to the field conditions. Each population consisted of 60 plants which were planted within a single row, at a distance of 60 cm × 60 cm. The analysed populations had good persistence of field conditions. Plant material was harvested and plant parts were separated.

Preparation of samples for isoflavones analysis. Bulk samples (3 per each population) were prepared for isoflavone analysis. Each bulk sample consisted of 3–5 single plants harvested at the full flowering stage. Plant material was air dried in a thin layer in the shadow, at the temperature of 18–22 °C and the air humidity of 55–65%. The drying process was regularly controlled and the damaged or discoloured samples were removed.

The drying process lasted for 7 days and, after its completion, the plant material was ground (polymix

Table 1. Origin of biological material: species, population code, latitude, longitude, and altitude

Species	Code	North latitude	East longitude	Altitude (m a.s.l.)
T. pratense	CS118	43°26.4881'	20°53.0006'	1184.5
	ES043	43°48.1530'	21°45.3527'	443.8
	ES077	44°08.3621'	21°58.6408'	758.8
	ES086	44°10.0418'	21°57.0274'	843.0
	RA100	43°17.5624'	20°01.7826'	1008.1
	RA123r	43°16.1972'	20°13.4084'	1233.0
T. pannonicum	CS105	43°16.1452'	20°52.3682'	1040.3
	CS119	43°26.5037'	20°53.0167'	1185.2
	CS146	43°18.9151'	20°51.2528'	1475.3
	ES047	43°49.8262'	22°03.6603'	478.4
	ES059	43°50.5721'	21°40.5952'	593.5
	RA123h	43°16.1972'	20°13.4084'	1233.0

T. pratense – *Trifolium pratense* (red clover); *T. pannonicum* – *Trifolium pannonicum* (Hungarian clover)

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systeme px-mfc 90 D; Kinematica AG, Switzerland) and stored in the dark stained glass jars. The isoflavone analysis was performed at the Department of Pharmacy, the Faculty of Medicine of the University of Novi Sad, Republic of Serbia.

Isoflavone extraction. The dry material was milled by a laboratory grinder (polymix systeme px-mfc 90 D; Kinematica AG, Switzerland). The plant material (1 g) was mixed with 2 mL of water and incubated (water bath, Julabo TW 20; Julabo GmbH, Germany) for 30 min at 37 °C. After that, 2 mL of 3M HCl and 16 mL of 96% ethanol were added, and the mixture was heated and boiled (laboratory heating device GP 1300; PC King, Serbia) for 10 min. The obtained extracts were filtered and purified by solid phase extraction. The Agilent SampliQ OPT cartridges were conditioned with 3 mL of methanol and 3 mL of water. Sample extract (0.5–1 mL) with 2 mL of water was added. Impurities were washed out with 3 mL of 5% methanol, and isoflavones were eluted with 3 mL of 80% methanol, 2 mL of 90% methanol, and 5 mL of pure methanol (Klejdus et al. 1999). Before HPLC injection, each extract was filtered using Agilent technologies Teflon filters (0.45 µm; USA).

HPLC analysis. The Zorbax SB-C_{18} column (250 × 4.6 mm) with 5-µm particles was used for the separation of the isoflavones. The mobile phase consisted of water, adjusted with sulfuric acid to pH 2.7 (A) and acetonitrile (B). The gradient profile was: 0–35 min from 20 to 37% B, 35–45 min from 37 to 100% B, 45-50 min 100% B, 50–51 min 100 to 20% B, 51–61 min 20% B, with 15 min post time. The flow-rate was 1 mL min^{-1}. The wavelength of detection was 254 nm. The injection volume was 10 µL and the column operated at room temperature (Krenn et al. 2002). Isoflavones were identified and quantified using five-point calibration curves ($r \geq 0.999$) and UV spectra of corresponding standard compounds (daidzein, genistein, formononetin, biochanin A). Standards were dissolved in 80% ethanol.

Chemicals. The following chemicals were used in the experiment: acetonitrile (J.T. Baker, Netherlands), 96% methanol (Zorka farm, Serbia), sulphuric acid (RTB, Serbia), 35% hydrochloric acid (POCH, Poland), ultra-pure water (TKA purification system 05.30C 7-DEN; Serbia). Standard compounds, the daidzein, and genistein were obtained from ChromaDex (USA) while formononetin and biochanin A were purchased from Sigma-Aldrich (USA).

Statistical analysis. All figures were created using Microsoft Excel 2016. All statistical analyses were performed using the Statistica 12.5 software (StatSoft, USA). One-way ANOVA followed by post-hoc comparison using Tukey's test was used to identify differences between populations in isoflavones content. Differences among treatments were considered at $P < 0.05$. Principal component analysis (PCA) was applied for determination of a general pattern in the measured variables.

RESULTS AND DISCUSSION

The average isoflavones content of the aerial plant parts in the studied red clover populations was 1.393 mg g^{-1}, while the content in Hungarian clover was 0.487 mg g^{-1} of air dry matter (Table 2), indicating that red clover has almost three times higher isoflavones content than the Hungarian clover. These results are consistent with the studies of Butkutė et al. (2014) who also found significantly higher isoflavones content in red clover. Except for the genistein, whose content is almost identical in both species (approximately 0.2 mg g^{-1} of air dry matter), the content of all other isoflavones was significantly lower in Hungarian clover.

The content of formononetin was very low (only 4%), in contrast to the high amount of biochanin A in both species (40% and 46% in Hungarian and red clover, respectively), see Figure 1. Since the genistein has approximately ten times higher biological activity (positively directed) than daidzein (Morito et al. 2001) the fact that it prevails in Hungarian clover makes it a desirable nutritive diet for animals. High levels of formononetin and biochanin A have been already reported (Sivesind and Seguin 2005; Oleszek et al. 2007). This amount of isoflavones was not equally distributed in the plant parts. The highest total isoflavones content was present in the red clover leaf, while the leaf and flower total isoflavones ratio was almost the same in Hungarian clover (Figure 2). Previous studies differ in speculations about the red clover part of the highest isoflavone content. Some indicated the flowers and

Table 2. The average isoflavone values of all analysed populations of red (*T. pratense*) and Hungarian clover (*T. pannonicum*) (mg g^{-1} of air dry matter)

Species	Daidzein	Genistein	Formononetin	Biochanin A	Σ
T. pannonicum	0.062	0.210	0.018	0.197	0.487
T. pratense	0.160	0.206	0.381	0.646	1.393

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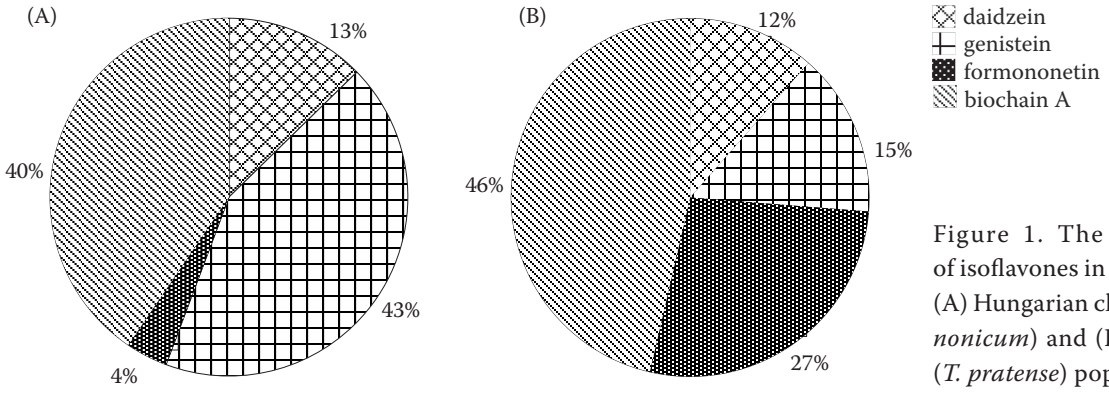

Figure 1. The percentage of isoflavones in the observed (A) Hungarian clover (*T. pannonicum*) and (B) red clover (*T. pratense*) population pool

leaves (Dabkevičienė et al. 2012), or leaves primarily (Bursać et al. 2011; Lemežienė et al. 2015). Concerning Hungarian clover, our results are partly consistent with Dabkevičienė et al. (2012). The highest and smallest content of isoflavones in our study was determined in flowers and stems, respectively.

The prevalence among isoflavones in flowers of both species was determined for biochanin A (Figure 3), daidzein, and genistein were significantly higher in Hungarian clover flowers, while formononetin content was much higher in flowers of red clover. The high content of genistein in the leaf and stem of Hungarian clover is consistentwith the results of Butkutė et al. (2014).

In the leaves of red clover, the biochanin A prevailed (1.206 mg g^{-1} on average), while the other isoflavones were significantly lower. The genistein was found as the most present isoflavones in the Hungarian clover leaves.

The highest content of formononetin and biochanin A was found in the red clover stem. The other two isoflavones were present in much lower concentrations. In the Hungarian clover stem, the presence of genistein and biochanin A was determined, while the formononetin concentration was in traces in addition to very low daidzein concentration.

Concerning differences among surveyed populations, the content of researched isoflavones showed that there were significant differences, dependent on the genotypic variations. It was shown that some red clover populations, such as ES086 and ES043 exhibited a high content of biochanin A and formononetin, in difference to low content of these isoflavones in populations assigned as CS118 and ES077 (Table 3). Populations of red clover did not differ significantly in daidzein and genistein content, whereas ES086 population exhibited the highest concentration of formononetin, biochanin A, and total isoflavones.

Populations of Hungarian clover also exhibited significant variations, where populations assigned as CS146, ES047, and RA123h showed the highest content of the total isoflavones (Table 4).

Variability of isoflavone concentration: Plant part and genotype-dependent variability. The total percentage of variability shown by the first two PCA axes was 83.8%. The isoflavone vectors were negatively correlated with the *x*-axis (Figure 4). The leaves of red clover much differed in the content of isoflavones compared with the other studied plant parts. However, the leaves were not regularly grouped, since the populations ES043 and ES086 are positioned

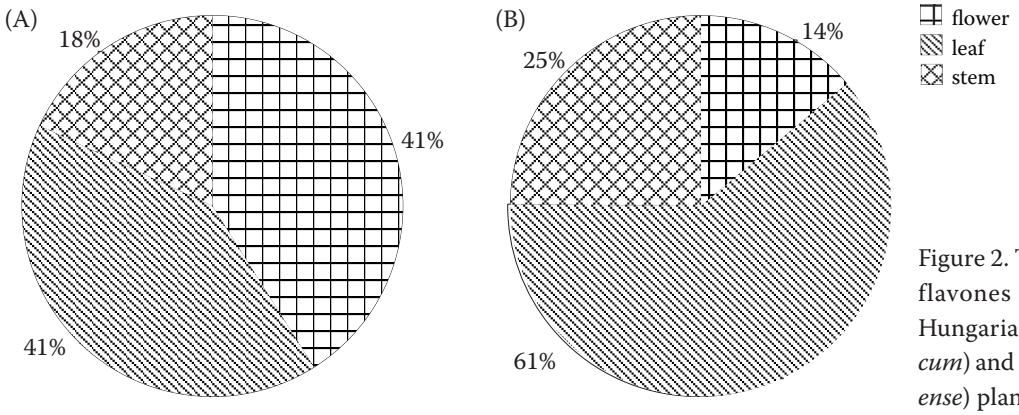

Figure 2. The percentage of isoflavones in the observed (A) Hungarian clover (*T. pannonicum*) and (B) red clover (*T. pratense*) plant parts

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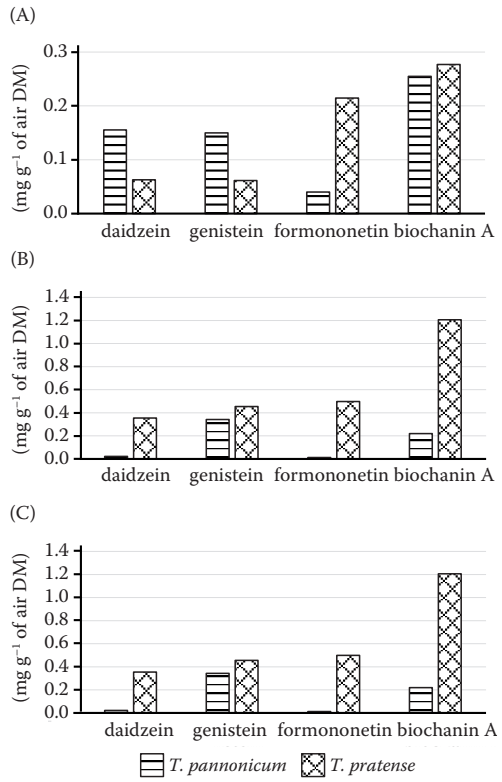

Figure 3. The distribution of individual isoflavones by plant parts: (A) flower, (B) leaf and (C) stem

DM – dry matter

differently, due to much higher formononetin and biochanin A content. The position of populations of RA123r and RA100 was mostly determined by high daidzein and genistein content.

On the Figure 4 there was a selection of the CS146 population flower and the RA123h population leaf on the left side. Their position was most influenced by the content of genistein.

Hungarian clover had generally lower content of isoflavones than red clover, but the species is known for a satisfactory amount of its crude proteins; the species is scharacterised by longevity and it positively influences the soil structure. Having in mind a range of desirable nutrient features in addition to the current assessment of major flavonoids, the isoflavones, different genotypes and varieties could be used in multi-component grass – legume mixtures for extensive exploitation. As stated by Polak and Jancova (2005), simplified nutrition in livestock leads to disorders and therefore the multi-component fodders and grazing grasslands of higher floristic biodiversity have a very positive impact on the animals.

The selected populations of red clover could be used in the process of breeding, to keep the presence of isoflavones in the future offspring, which will have health promoting effect on animals.

Table 3 Mean values of daidzein, genistein, formononetin, and biochanin A by the population of *T. pratense* (mg g^{-1} of air dry matter; mean errors and LSD test with a significance threshold of $P < 0.05$)

Population	Daidzein	Genistein	Formononetin	Biochanin A	Total
CS118	0.081 ± 0.091^{a}	0.185 ± 0.233^{a}	0.088 ± 0.064^{c}	0.252 ± 0.177^{c}	0.606 ± 0.423^{c}
ES043	0.129 ± 0.084^{a}	0.141 ± 0.155^{a}	0.719 ± 0.612^{a}	0.971 ± 0.866^{ab}	2.127 ±1.964^{ab}
ES077	0.123 ± 0.070^{a}	0.262 ± 0.133^{a}	0.245 ± 0.063^{bc}	0.308 ± 0.061^{c}	0.937 ± 0.219^{c}
ES086	0.207 ± 0.159^{a}	0.106 ± 0.042^{a}	0.778 ± 0.235^{a}	1.343 ± 1.043^{a}	2.770 ± 1.574^{a}
RA100	0.227 ± 0.290^{a}	0.191 ± 0.222^{a}	0.101 ± 0.053^{bc}	0.424 ± 0.434^{bc}	0.944 ± 0.885^{c}
RA123r	0.193 ± 0.232^{a}	0.350 ± 0.419^{a}	0.357 ± 0.181^{b}	0.578 ± 0.238^{bc}	1.478 ± 0.910^{bc}

LSD – least significant difference; $^{a–d}$Different letters within columns indicate significant differences in mean values ($P < 0.05$)

Table 4. Mean values of daidzein, genistein, formononetin, and biochanin A by population of *T. pannonicum* (mg g^{-1} of air dry matter; mean errors, and LSD test with a significance threshold of $P < 0.05$)

Population	Daidzein	Genistein	Formononetin	Biochanin A	Total
CS105	in traces^{a}	0.130 ± 0.044^{bc}	0.010 ± 0.015^{a}	0.156 ± 0.08^{bc}	0.296 ± 0.12^{cd}
CS119	0.024 ± 0.036^{a}	0.222 ± 0.055^{abc}	0.015 ± 0.022^{a}	0.175 ± 0.026^{bc}	0.436 ± 0.068^{bcd}
CS146	0.215 ± 0.323^{a}	0.252 ± 0.048^{ab}	0.042 ± 0.062^{a}	0.229 ± 0.075^{ab}	0.738 ± 0.434^{a}
ES047	in traces	0.239 ± 0.158^{ab}	in traces	0.321 ± 0.271^{a}	0.560 ± 0.290^{abc}
ES059	0.037 ± 0.055^{a}	0.091 ± 0.026^{c}	in traces	0.094 ± 0.037^{c}	0.222 ± 0.044^{d}
RA123h	0.095 ± 0.038^{a}	0.325 ± 0.303^{a}	0.044 ± 0.026^{a}	0.208 ± 0.092^{abc}	0.672 ± 0.445^{ab}

LSD – least significant difference; $^{a–d}$Different letters within columns indicate significant differences in mean values ($P < 0.05$)

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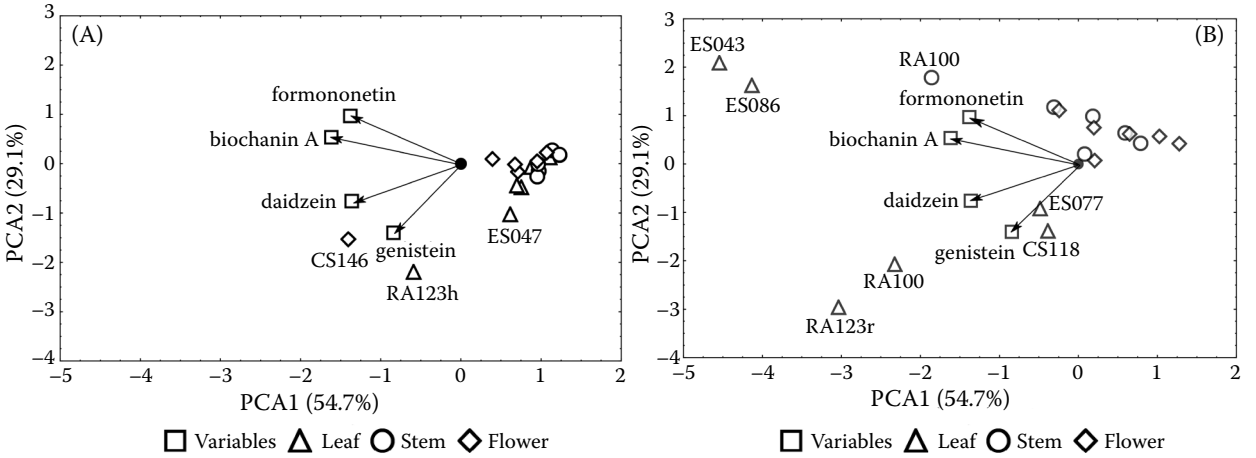

Figure 4. PCA score and loading biplot based on the isoflavones content in the leaves, flowers, and stems of different (A) Hungarian (*T. pannonicum*) and (B) red clover (*T. pratense*) populations

PCA – principal component analysis

In the soybean breeding experiment on isoflavones inheritance, it was showed that there is an offspring in the total content and composition of isoflavones from parental to F1 generation (Cvejić et al. 2011).

CONCLUSION

Wild populations of red clover exhibit a much higher content of isoflavones than populations of Hungarian clover. The genistein was the most prevailed isoflavone, in Hungarian clover (43%), while red clover populations were very rich in biochanin A (46%). The distribution of isoflavones in plant parts differed significantly. Red clover accumulated the highest level of isoflavones in the leaf, whereas Hungarian clover populations exhibited mostly equal levels of isoflavones, in leaves and flowers. The analysis of variance showed that there were statistically significant differences among natural populations and between the species. As the analysed material exhibited significant starting variability, the criteria of the breeding process for enhanced content of the total and individual isoflavones should be concerning in relation with primary nutrients' composition and overall fodder quality, especially in light of the impact on animal health benefits.

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