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Karakterizacija sadržaja izoflavona u hrvatskoj kolekciji crvene djeteline

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THE CHARACTERIZATION OF ISOFLAVONE CONTENT IN THE CROATIAN RED CLOVER COLLECTION

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SUMMARY

Red clover is a perennial forage crop rich in isoflavones, the bioactive compounds with a positive effect on human and animal health. The aim of the research was to determine a variation in the isoflavone contents in the Croatian red clover collection and to identify the most promising materials to be used in the breeding program for the development of new cultivars for specific purposes. Leaf samples of 29 red clover cultivars/populations (two cultivars, twenty breeding populations, and seven local populations) were collected in a full flowering stage, and the identification and quantification of isoflavones was performed using the HPLC analysis. The most common isoflavones in the red clover cultivars/populations were formononetin and biochanin A. A significant variation among the cultivars/populations in the total and individual isoflavone content was determined. The populations with very high and low contents of both the total and of the individual isoflavones were identified to be used for the breeding purposes in order to develop new forage cultivars, or for specific goals in the pharmaceutical industry.

Key words: red clover, cultivars/populations, isoflavones, formononetin, variation, HPLC analysis

INTRODUCTION

Red clover (*Trifolium pratense* L.) is one of the main fodder species in grassland agroecosystems worldwide. It is a short-term perennial legume that grows for about two to three years and is adapted to a wide range of climatic conditions, soil types, fertility levels, use patterns and management strategies. Red clover has a high yielding potential and cultivars grown in monoculture in humid climatic conditions can achieve in the second seasons over 80 t ha^{-1} of green mass yield and 15 t ha^{-1} of dry matter yield (Tucak et al., 2016). Red clover provides a high protein-rich feed for grazing livestock and during conservation is prone to the lower levels of protein degradation, compared to other legume feed sources (Phelan et al., 2015; Hart et al., 2016). The importance of red clover in agricultural production, as well as its positive impact on environmental conservation, are the known facts and are often studied in numerous scientific investigations (Vasilev et al., 2005). This forage legume is also a rich natural source of bioactive compounds, which can have positive and/or negative effects on human and animal health.

Its use as an alternative source of isoflavones became interesting for the scientific community over the last few decades because of the several key reasons, such as the following ones: a possibility of sustainable and environmentally friendly production;

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a limited use of soybean, presently a major source of isoflavonoids in human nutrition due to the allergenic reactions it can cause in some people; a steady increase in the production area of genetically modified soybean notwithstanding a fact that many people and scientists do not approve the use of such plants (Aparicio-Fernandez et al., 2005; Saviranta et al., 2008; Dabkevičiene et al., 2012; Butkute et al., 2014; Tucak et al., 2018).

The main group of bioactive compounds detected in the red clover are isoflavones. The amounts of isoflavones in the red clover are the highest in leaves, followed by the stems, petioles, and flowers (Sivesind and Seguin, 2005; Bursać et al., 2011; Du et al., 2012; Lemeziene et al., 2015; Butkute et al., 2018). About 40 different isoflavones are detected in the red clover, of which the most common are formononetin, biochanin A, daidzein and genistein (Butkute et al., 2014; Tava et al., 2015; Hloucalova et al., 2016; Kumar et al., 2018; Tava et al., 2019). Isoflavones are the secondary plant metabolites and constitute one of the most common categories of phytoestrogens, having an ability to cause oestrogenic and/or anti-oestrogenic effects (Kowalska et al., 2013; Daems et al., 2016; Bustamante-Rangel et al., 2018). Numerous clinical reports have suggested a positive effect of isoflavones in human health and nutrition, such as a decreasing risk of heart disease, moderation of menopausal symptoms, prevention of some forms of cancer, cardiovascular diseases and osteoporosis (Lipovac et al., 2012; Gartoulla and Ham, 2014; Shakeri et al., 2015; Cornara et al., 2016; Kolodziejczyk-Czepas, 2016). Isoflavones are also of a scientific interest because of their biological activity exerted on the farm animals' health, on which they may have both the positive and the negative effects.

A consumption of feed containing the large amounts of isoflavones, especially formononetin, has been proven to cause the temporary or permanent fertility problems in sheep and cows (Sivesind and Seguin, 2005; Mustonen et al., 2014; Little et al., 2017; Mustonen et al., 2018). However, animal feeding experiments have demonstrated that the red clover diets (grazing pasture or silage) with a high isoflavone concentration (including daidzein and formononetin) promote the growth and increase a live-weight gain in sheep and lambs, and cause a great increase in the cows' milk equol content (Moorby et al., 2004; Woclawek-Potocka et al., 2013; Kalač, 2013; Mustonen et al., 2018). The milk from dairy cows, therefore, appears to be a potential source of equol in human nutrition (Kalač, 2011; Tsen et al., 2014).

Despite the current importance of red clover as a source of isoflavones for the nutraceutical industry, as well as its widespread use as a feed for farm animals, there has been no comprehensive research on the isoflavone content in the Croatian red clover cultivars and populations. The objective of present investigation was to determine a variation in the isoflavone contents of red clover collection and to identify most promising materials to be used for our further breeding activities and development of new cultivars for specific purpose.

MATERIAL AND METHODS

Plant materials and field experiments

Twenty-nine diploid red clover cultivars/populations from a broad range of origins, including two cultivars, twenty breeding populations, and seven local populations created at the Department of Forage Crops Breeding and Genetics, Agricultural Institute Osijek, Croatia, were evaluated in this study (Table 1). A field experiment was established in the spring of 2014 at the Agricultural Institute Osijek. The plots were arranged as a randomized complete block design with four replications. The seed of each cultivar/population was planted at a rate of 18 kg ha^{-1} in plots measuring 1.4 x 6 m. The experiment was not irrigated, fertilized, or protected against weeds/pests/diseases.

Weather conditions at the experimental site

The average monthly air temperatures during the red clover growing season were higher in all months, except in May and August, compared to the long-term monthly average temperatures. In 2014, the average air temperature was higher by 1.1°C, compared to the long-term average temperatures (Figure 1A). A higher total amount of precipitation was recorded in all months of the studied year if compared to the long-term average, excluding March and August, when the total amount of precipitation was slightly higher during the long-term average (Figure 1B). The particularly rainy month in 2014 was May, which had 63% more precipitation, compared to the same month of the long-term average.

Also, significantly more precipitation was recorded during the growing season (650.6 mm) of 2014, by 28% more in comparison to the long-term average (467.8 mm), which indicates that weather conditions during the red clover growing season of 2014 were much more humid and moderately warm.

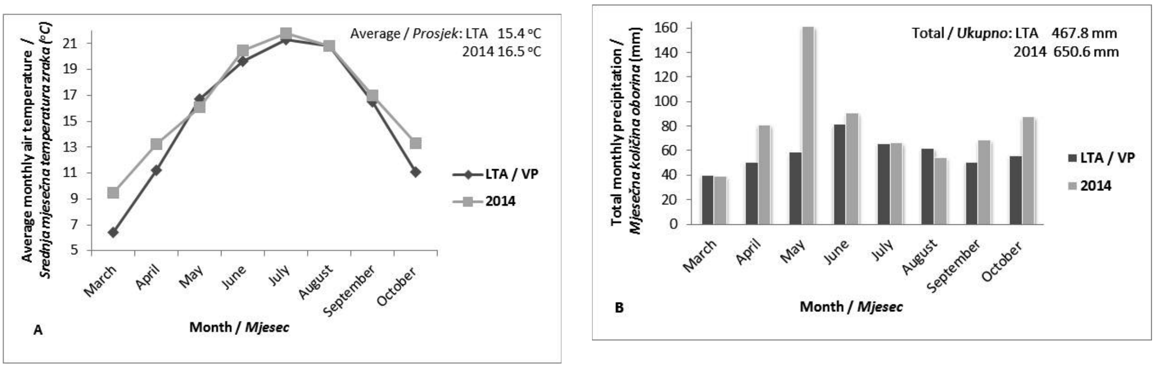

Figure 1.A.B. Average monthly air temperatures and total monthly precipitation for the red clover growing season (March - October) during the study period (2014) and a long-term average (1971-2000) for the Osijek experimental site.

Slika 1.A.B. Prosječne mjesečne temperature zraka i ukupne mjesečne padaline za vegetacijsko razdoblje (ožujak - listopad) crvene djeteline tijekom provedbe istraživanja (2014.) i višegodišnji prosjek (1971.-2000.) za pokusnu lokaciju Osijek.

Sample preparation

In the second cut of the red clover in a full flowering stage (second half of July 2014), the average leaf samples (i.e., the healthy, young, and fully developed ones) from two replications of cultivars/populations were randomly collected from the plants in the middle of each plot. The collected leaf samples were stored in a refrigerator (-80°C), lyophilized, and ground into a fine powder by an oscillating mill immediately before extraction.

Isoflavone analysis

Extraction

The extraction of isoflavones was performed according to the partially modified procedure of Ramos et al. (2008), as follows: the homogenized red clover samples (0.05 g) were extracted with 4 ml of hydrochloric acid (6M HCl) and incubated in a water bath for 15 mins. at 100°C, with occasional vortexing. After cooling, the extracts were filtered, and the residue was washed with 5 ml of MeOH. The purified samples were filtered to a volumetric flask up to 10 ml and diluted to volume with distilled water, being centrifuged thereafter for 10 mins. at 4000 rpm. The obtained extracts were kept at -21°C until a HPLC analysis (high-performance liquid chromatography) was performed. For each sample, one extract was prepared for analysis in two parallels. Prior to the HPLC analysis, the obtained extracts were filtered through a 0.45-μm pore size membrane filter.

Chromatographic conditions

Chromatographic separations of isoflavones were performed by the HPLC system (Perkin Elmer LC 200 Chromatograph) on a RP-C18 column (Nova-Pak, 4 um, 3.9*150 mm). The mobile phase solvents A (water:trifluoroacetic acid (H_2O/0.01% TFA) and B (acetonitrile:trifluoroacetic acid (ACN/0.1% TFA) were used. Isoflavone substances were separated by injection of 10 μl of extract with a linear ACN gradient of 25 to 50% for 15 min, with a flow-rate of 1 ml/min, at a column temperature of 30°C and at a wavelength of 260 nm (a DAD detector).

Identification and quantification

The identification (based on a retention time and the UV spectra of substances) and quantification (the formation of calibration curves in the considered substance concentration ranges) of isoflavones were performed using the TotalChrom Software Package and standard solutions of daidzein, genistein, formononetin and biochanin A. Figure 2 shows a HPLC-DAD chromatogram of the isoflavones identified in red clover leaves extract.

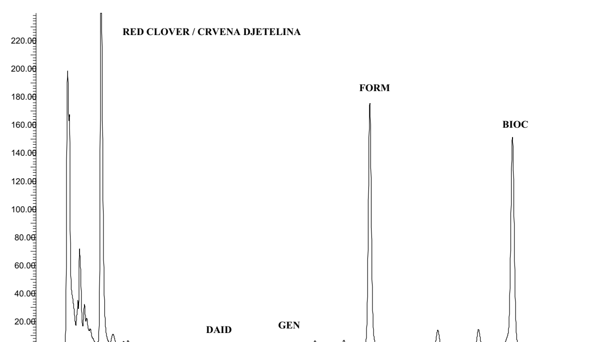

Figure 2. HPLC-DAD chromatogram of isoflavones identified in the red clover leaf extract (DAID - Daidzein, GEN - Genistein, FORM - Formononetin, BIOC - Biochanin A).

Slika 2. HPLC-DAD kromatogram izoflavona identificiranih u ekstraktu lišća crvene djeteline (DAID - Daidzein, GEN - Genistein, FORM - Formononetin, BIOC - Biochanin A).

Statistical analysis

The experimental data were statistically processed using the STAR v. 2.0.1 (IRRI, 2013) software. The data were subjected to analysis of variance (ANOVA) to enable a LSDs calculation. Fisher's protected LSD test was used at the 0.05 and 0.01 probability level to identify significant differences between the mean values of cultivars/populations. The STATISTICA v8.0 (StatSoft, Inc., Tulsa, OK, USA) statistical toolset was utilized for a principal component analysis. For the first two principal components, an explained variation among all the red clover cultivars/populations was graphically represented in a scatter plot.

RESULTS AND DISCUSSION

The most abundant isoflavone in the red clover cultivars/populations was formononetin, with a share of 61.81% in the total amount of isoflavones. A slightly lower content was determined for biochanin A (36.12%), while the presence of daidzein and genistein (0.80% and 1.26%) in the total amount of isoflavones of all red clover cultivars/populations was significantly lower (Figure 3). The highest accumulation of formononetin and biochanin A in both the whole plant and/or in the individual plant parts at a red clover flowering stage was confirmed by the studies of numerous authors (Tsao et al., 2006; Butkute et al., 2014; Lemeziene et al., 2015; Hloucalova et al., 2016; Butkute et al., 2017; Little et al., 2017; Mustonen et al., 2018). Besides formononetin and biochanin A, Tava et al. (2015; 2019) reported the high amounts of other flavonoids, such as quercetin derivatives, in the red clover leaves and flowers.

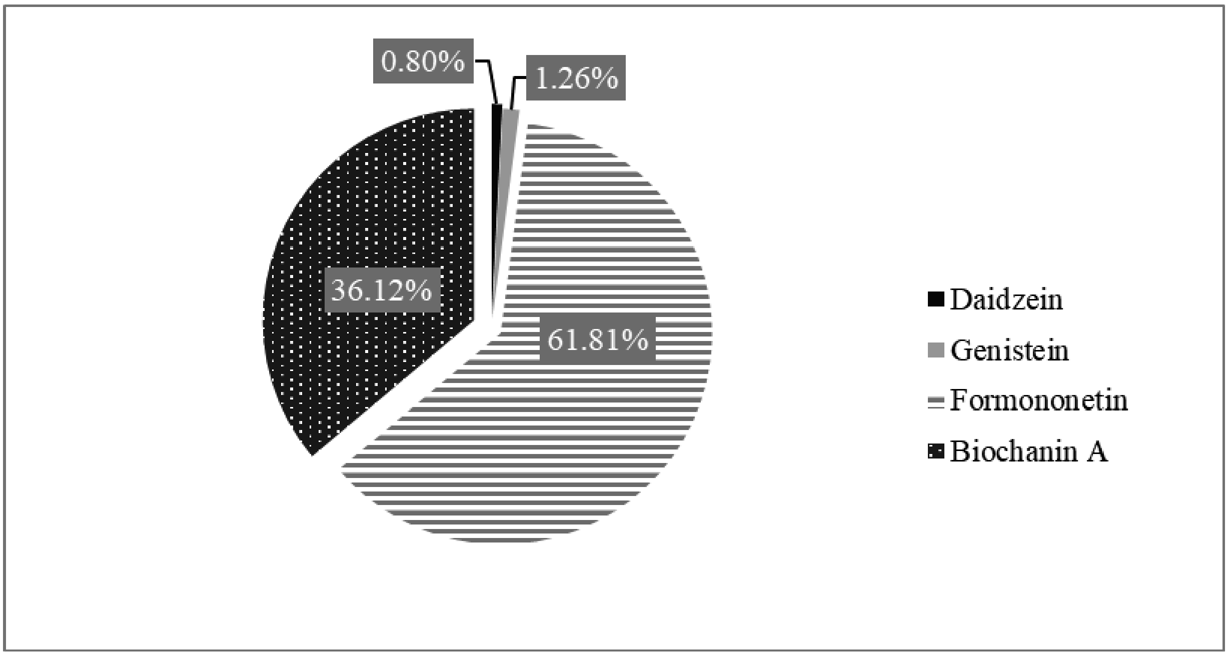

Figure 3. Proportion (%) of individual isoflavones in the total amount of isoflavones of all red clover cultivars/ populations at a full flowering stage (second half of July 2014).

Slika 3. Udio pojedinih izoflavona (%) u ukupnoj količini izoflavona svih sorti/populacija crvene djeteline u fazi punog cvjetanja (druga polovica srpnja, 2014.).

Analysis of variance demonstrated a statistically significant variation between the red clover cultivars/ populations in the content of all individual isoflavones, as well as in the content of total isoflavones (Table 1). The main isoflavones in the red clover cultivars/ populations were formononetin, varying in the range of 3672.77-9883.30 μg g^{-1} of dry matter (DM), followed by biochanin A, varying in the range of 1811.01-6123.70 μg g^{-1} of DM. The respective contents of genistein, 54.13-318.30 μg g^{-1} of DM, and daidzein, 43.46-114.82 μg g^{-1} of DM, were considerably lower than the contents of the main isoflavones. The total content of isoflavones in 29 evaluated red clover cultivars/populations ranged between 6575.61-11847.43 μg g^{-1} of DM. The breeding population CD-6 had the highest content of formononetin and total isoflavones, as well as a high content of daidzein. The high levels of total isoflavones and most of the individual isoflavones were also found in breeding populations CD-8, CD-1, Rc-15/10 and Rc-13/23. The lowest content of total isoflavones was determined in the breeding population Rc-15/6, and was lower by as much as 44.49%, compared to the CD-6 breeding population, which had the largest accumulation of total isoflavones. The breeding population Rc-15/6 also featured the lowest content of genistein and the low levels of biochanin A and daidzein. The lowest content of formononetin and daidzein was found in local population Rc-13/51, which also had a low content of total isoflavones. Low levels of total isoflavones and most of the individual isoflavones were also determined in the breeding populations Rc-13/40, Rc-15/12, and Rc-15/7.

Table 1. Average isoflavone content (μg g^{-1} of dry matter) in the leaves of a red clover collection.

Tablica 1. Prosječni sadržaj izoflavona (μg g^{-1} suhe tvari) u listovima crvene djeteline.

No.	Cultivar/ Population *Sorta / Populacija*	Improvement status *Status materijala*	Daidzein	Genistein	Formononetin	Biochanin A	Total isoflavone *Ukupni izoflavoni*
1	CD-12	LP	77.55	102.68	5271.58	3330.65	8782.46
2	CD-13	LP	69.74	145.38	5423.12	3933.74	9571.97
3	PopMp	LP	93.01	95.86	6625.22	2682.86	9496.94
4	Rc-11/7	BP	63.02	107.89	4262.18	3141.85	7574.94
5	CD-1	BP	86.79	101.27	7078.56	3105.71	10372.33
6	CD-9	BP	75.04	138.13	4895.84	3605.72	8714.73
7	Rc-11/8	BP	85.46	126.27	4089.87	3081.99	7383.59
8	OS Viva	C	72.26	93.68	5904.34	2596.55	8666.84
9	Rc-11/13	BP	58.18	106.72	4489.07	2642.87	7296.85
10	CD-10	LP	66.75	128.02	5157.63	2580.77	7933.18
11	Rc-11/15	BP	66.30	101.78	5639.75	3013.02	8820.85
12	Rc-13/6	BP	58.76	89.08	4371.84	2333.71	6853.39
13	CD-11	LP	70.95	112.22	4261.58	2284.96	6729.70
14	Rc-13/19	BP	67.99	99.92	5095.99	2282.64	7546.55
15	Rc-13/23	BP	114.82	102.96	6543.66	2644.78	9406.22
16	Rc-13/24	BP	70.85	95.89	5006.20	2193.97	7366.91
17	Rc-13/25	BP	67.06	94.68	5257.95	2454.05	7873.73
18	OS Osiris	C	55.52	90.35	5254.66	2794.99	8195.52
19	Rc-13/40	BP	44.93	96.46	3796.26	2841.64	6779.29
20	Rc-13/51	LP	43.46	113.39	3672.77	3910.23	7739.85
21	Rc-15/6	BP	52.04	54.13	4506.19	1963.25	6575.61
22	Rc-15/7	BP	54.31	113.15	3798.39	6123.70	10089.55
23	Rc-15/9	LP	70.68	104.00	4698.07	4183.87	9056.62
24	Rc-15/10	BP	53.62	73.20	5142.06	5170.60	10439.49
25	Rc-15/12	BP	52.35	70.50	4751.54	2213.20	7087.59
26	Rc-15/21	BP	63.72	318.30	5012.47	2576.64	7971.13
27	CD-6	BP	87.48	65.63	9883.30	1811.01	11847.43
28	CD-7	BP	53.70	63.92	4241.99	2443.79	6803.40
29	CD-8	BP	67.39	85.53	6965.88	4359.00	11477.81
Average			67.71	106.59	5210.27	3044.89	8429.46
LSD 0.05			4.42	10.87	46.68	52.49	74.85
LSD 0.01			5.97	14.66	62.98	70.81	100.97

*Cultivar - C, Breeding population - BP, Local population - LP / *Sorta - C, Oplemenjivačka populacija - BP, Lokalna populacija - LP*

Our results showed great variation in the isoflavone content between the various red clover materials, which indicates an important effect of the genetic factor on their accumulation in the plant and is in accordance with the earlier reports by Sivesind and Seguin (2005), Papadopoulos et al. (2006), Little et al. (2017) and Mustonen et al. (2018). Ramos et al. (2012) evaluated a genetic variability of isoflavones in the red clover accessions of different origin and improvement status and determined a high significant variation with regard to the content of all individual and total isoflavones, as follows: 452.97-28548.65 μg g^{-1} of DM for formononetin, 2199.02-15670.39 μg g^{-1} of DM for biochanin A, 14.70-516.91 μg g^{-1} of DM for genistein, 0-137.91 μg g^{-1} of DM for daidzein, and 9810-36360 μg g^{-1} of DM for total isoflavones. Lemeziene et al. (2015) determined the isoflavone values in the Lithuanian red clover cultivars and ecotypes and found that their contents in the leaves ranged between 3980-7170 (formononetin), 4570-6868 (biochanin A), 490-720 (genistein), 60-170 (daidzein), and 9400-14370 (total) μg g^{-1} of DM. Mustonen et al. (2018) reported that the total content of isoflavones in the red clover cultivars varied in the range of 11200-14800 μg g^{-1} of DM, while the content of individual isoflavones formononetin, biochanin A, genistein and daidzein ranged between 5950-7890, 3660-6070, 490-550, and 230-300 μg g^{-1} of DM, respectively. The values measured in our research are similar to the results reported by Lemeziene et al. (2015) and Mustonen et al. (2018). Considerably higher contents of isoflavones compared to our values were obtained by Lemeziene et al. (2015). These widely different results may be explained by the fact that a total isoflavone content and the content of individual isoflavones depend on many other factors in addition to a cultivar, such as a farm management, harvest timing and growing seasons, plant phenological stages, plant parts, growth conditions, sampling methodology, climatic conditions, preservation method and storage. Temperature and water availability are the very important environmental factors affecting isoflavone levels. Generally, a short period of water stress has no effect on isoflavone content, and only a long-term progressive drought can modify the production of these compounds. Lower temperatures during the vegetative growth may increase the isoflavone content in the red clover and other species that are the rich sources of isoflavones, such as the subterranean clover and soybean. Weather conditions during the investigated period were humid, without a drought period, and moderately warm in comparison to the long term-average (Figure 1AB), and probably had favorable influence on the accumulation of isoflavones in the leaves of red clover cultivars/populations.

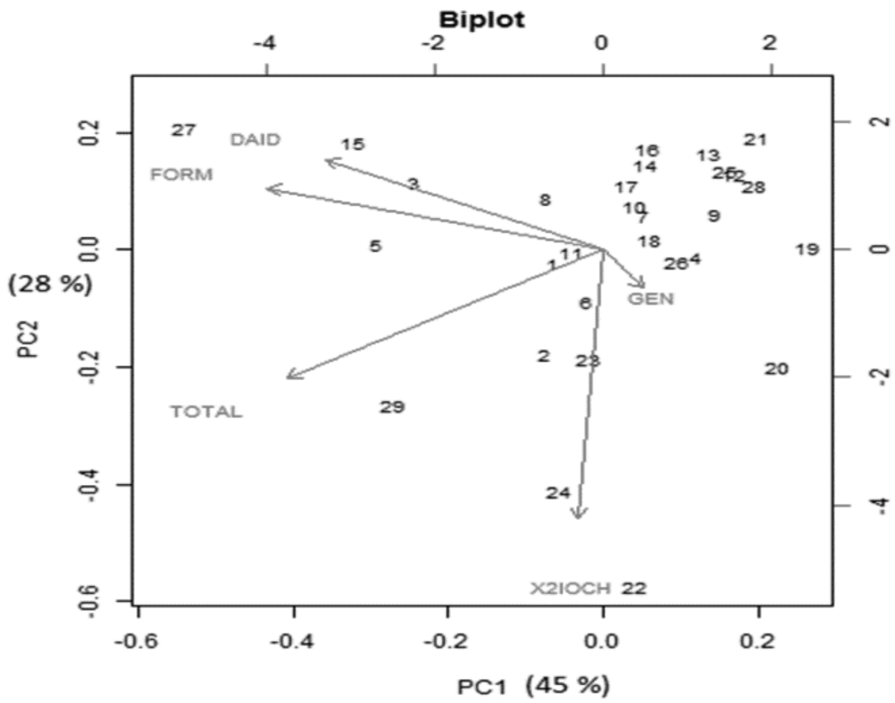

Figure 4. Biplot analysis for the content of isoflavones measured in 29 red clover cultivars/populations (in Table 1, see the description for 1-29).

Slika 4. Biplot analiza za sadržaj izoflavona izmjerenih kod 29 kultivara / populacija crvene djeteline (u Tablici 1. vidjeti opis za 1-29).

The main sources of genetic variation between the studied red clover cultivars/populations were determined using the Principal Components Analysis (PCA) (Figure 4). The first three principal components (PC 1, 2, and 3) gave eigenvalues greater than 1.0 and explained 90% of the total variation between the red clover cultivars/populations for all of the investigated isoflavones. PC 1, which is the most important component, accounted for 45% of the total variation and was associated with the formononetin content and the total isoflavone content. PC 2 explained 28% of the variation and was defined by the content of biochanin A. The biplot analysis showed that the largest number of red clover cultivars/populations clustered in three groups. The group with the largest number of populations located in the upper right part of the biplot was characterized by the mean values of the contents of most of the individual and total isoflavones. Most of the populations grouped on the left side from the centers had high content of total and individual isoflavones, while the populations with a low content of isoflavones were located in the right bottom part of the biplot. The biplot shows that there is a potential to identify the red clover populations with the high or with the low contents of isoflavones.

CONCLUSION

A significant variation in contents of individual and total isoflavones was found among the investigated red clover cultivars/populations. The most dominant isoflavone in the observed red clover materials was formononetin. The identified populations with a high (CD-6, CD-8, CD-1, Rc-15/10, Rc-13/23) and low (Rc-15/6, Rc-13/51, Rc-13/40, Rc-15/12, Rc-15/7) content of isoflavones represent a new source of germplasm for the progress of our breeding activities and the development of cultivars for specific purposes and the use in livestock or pharmaceutical industries.

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KARAKTERIZACIJA SADRŽAJA IZOFLAVONA U HRVATSKOJ KOLEKCIJI CRVENE DJETELINE

SAŽETAK

Crvena djetelina višegodišnja je krmna kultura bogata izoflavonima, bioaktivnim spojevima s pozitivnim utjecajem na zdravlje ljudi i životinja. Cilj istraživanja bio je utvrditi varijabilnost sadržaja izoflavona hrvatske kolekcije crvene djeteline te identificirati najzanimljivije materijale koji će se koristiti u oplemenjivačkome programu za razvoj novih sorata za specifične namjene. Uzorci lista prikupljeni su u stadiju pune cvatnje iz 29 sorata/populacija crvene djeteline (dvije sorte, dvadeset oplemenjivačkih populacija, sedam lokalnih populacija), a identifikacija i kvantifikacija izoflavona obavljena je pomoću HPLC analize. Najzastupljeniji izoflavoni u sortama/populacijama crvene djeteline bili su formononetin i biohanin A. Utvrđena je značajna varijabilnost među sortama/populacijama u sadržaju ukupnih i individualnih izoflavona. Identificirane su populacije s vrlo visokim i niskim sadržajem kako ukupnih, tako i pojedinačnih izoflavona koje će se koristiti u oplemenjivačke svrhe za razvoj novih krmnih kultivara ili za specifične namjene u farmaceutskoj industriji.

Ključne riječi: crvena djetelina, sorte/populacije, izoflavoni, formononetin, varijabilnost, HPLC analiza

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