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The concentration of isoflavones in red clover (*Trifolium pratense* L.) at flowering stage

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Abstract

The objective of this study was to determine the concentrations of isoflavones in the cultivars and ecotypes of red clover (*Trifolium pratense* L.) in a whole above ground part of a plant and separately in stems, leaves and flowers at flowering stage. Isoflavones were extracted using acidified aqueous methanol and subsequent analyses of the extracts were carried out by ultra-performance liquid chromatography coupled with a photodiode array detector. Red clover accumulated the highest concentrations of formononetin (51%) and biochanin A (40%) at flowering stage. Concentrations of formononetin, biochanin A, daidzein and genistein at flowering stage ranged between 2.61–4.40, 1.79–3.32, 0.06–0.14 and 0.36–0.59 mg g⁻¹ dry matter (DM), respectively. The average concentration of all four isoflavones at flowering stage during two years of investigations was 6.66 mg g⁻¹ DM and ranged from 5.4 to 8.09 mg g⁻¹ DM. The analysed genotypes formed the following sequence according to total isoflavone concentration mg g⁻¹ DM: ‘Radviliai’ (8.09) > ‘Vyliai’ (7.29) > No. 2739 (7.15) > No. 2331 (6.92) > ‘Kiršiniai’ (6.90) > No. 2177 (6.47) > ‘Kamaniai’ (6.41) > ‘Arimaičiai’ (6.28) > ‘Sadūnai’ (6.17) > ‘Vyčiai’ (6.11) > ‘Liepsna’ (5.44).

The highest concentration of isoflavones at flowering stage was accumulated in leaves. The average total concentration of all four isoflavones was as follows: 12.29 mg g⁻¹ DM in leaves, 2.93 mg g⁻¹ DM in stems and 1.42 mg g⁻¹ DM in flowers. Plant material of all the tested cultivars and wild ecotypes, especially leaves of commercial diploid cv. ‘Radviliai’, tetraploid cv. ‘Vyliai’ and semi-natural ecotype No. 2739 containing the highest concentration of isoflavones, could be used for dietary supplement production in the pharmaceutical industry as a source of isoflavones.

Key words: above ground part, bioactive compounds, biochanin A, daidzein, formononetin, genistein.

Introduction

The main group of bioactive compounds detected in red clover is isoflavones. There are approximately 40 different isoflavones in red clover (Kjeldus et al., 2001; Rijke de et al., 2004). The main isoflavones are formononetin, biochanin A, daidzein and genistein. Isoflavones are natural substances which elicit a number of physiological effects in living organisms (Vacek et al., 2008). Biotic and abiotic stresses are known to induce the accumulation of bioactive compounds in plants (Jaganath, Crozier, 2009).

As any bioactive compound, isoflavones can have both positive and negative effects on animal health. Negative effect is primarily associated with animal reproduction. It was established that high concentrations of formononetin increase the number of abortions and stillbirths in ewes (Sakakibara et al., 2004). Therefore, it is not recommended to feed animals on diets rich in isoflavones during mating season and early pregnancy (Sakakibara et al., 2004; Woźlawek-Potocka et al., 2013).

However, phytoestrogens do not only have negative effects. Moorby et al. (2004) found that lambs grazing red clover with high concentrations of formononetin had slightly higher daily weight gains than lambs grazing red clover with lower formononetin concentrations or perennial ryegrass, with the same daily dry matter intake. Additionally, phytoestrogens, when red clover containing diets (grazing pastures or silage) are fed to dairy cows are partly transferred to their milk (Höjer et al., 2012; Adler et al., 2014).

Red clover has been used as food since old times as well. Without any knowledge of nutritious value or existence of bioactive compounds people felt positive effect of these *Fabaceae* plants on health. There is evidence that leaves and young plants of red clover have been used fresh (in salads) or thermally processed for teas since ancient times (Dénes et al., 2012; Łuczaj, 2012; Svanberg, 2012). During the recent decades, quite a few investigations have been carried out to

establish the nutritious value and bioactive compounds in some *Fabaceae* plant species (including red clover) (Saviranta et al., 2008; Gawel, 2012; Ince et al., 2012; Lindström et al., 2014). It was found that phytoestrogens isoflavones present in the biomass of above ground part of red clover can prevent different diseases, especially relevant to women (Atkinson et al., 2004; Miadokova, 2009; Mortensen et al., 2009).

The concentration of isoflavonoids mainly depends on several factors: plant development stage, genotype and structure of above ground parts of a plant – leaves, flowers and stems (Sivesind, Seguin, 2005; Tsao et al., 2006; Oleszek et al., 2007; Saviranta et al., 2008; Dabkevičienė et al., 2012).

In the literature, there are no comparative data on the concentration of isoflavonoids in different cultivars of red clover and its wild populations or differences between diploid and tetraploid cultivars. The concentration of

Table. Red clover germplasm collection (2013–2014)

No.	Cultivar / wild ecotype	Status	Ploidy level	Origin	The full flowering stage date	Dry matter yield kg m ⁻²
1.	Sadūnai	commercial cultivar	4n	Lithuania	27 06	0.38
2.	Vyčiai	commercial cultivar	2n	Lithuania	12 06	0.31
3.	Arimaičiai	commercial cultivar	2n	Lithuania	03 07	0.39
4.	Radviliai	commercial cultivar	2n	Lithuania	03 07	0.32
5.	Vyliai	old cultivar	4n	Lithuania	03 06	0.40
6.	Kamaniai	old cultivar	2n	Lithuania	09 07	0.33
7.	Liepsna	old cultivar	2n	Lithuania	12 06	0.23
8.	Kiršiniai	old cultivar	4n	Lithuania	12 06	0.29
9.	No. 2177	wild ecotype	2n	Lithuania	12 06	0.19
10.	No. 2331	wild ecotype	2n	Russia	12 06	0.27
11.	No. 2739	semi-natural ecotype	2n	Latvia	09 07	0.31

The germplasm collection was established in a field trial in 2012. The seed accessions were sown in one row (2.5 × 0.5 m) in four replications. Two replications were intended for the evaluation of agronomic traits, another two – for chemical analyses. The first cut for dry matter (DM) yield evaluation was taken at full flowering stage of each genotype, the second – after regrowth of aftermath. No herbicides were applied in the collection nursery.

The weather conditions of the experimental period markedly differed between plant vegetation seasons. The first season (2012) was characterized by an early spring, wet summer and warm and long autumn; the second season (2013) – by a very late, dry and warm spring and changeable summer with heat waves; the third season (2014) – by a warm summer (particularly July) and a long and rainy autumn.

Sample preparation and chemical analyses. For chemical analyses the plant samples were cut at full flowering stage in 2013 (first harvest year) and 2014 (second harvest year). Above ground part of a plant of each genotype, sampled in 2013 and in 2014 at flowering, was investigated each year separately (2013 – 1 sample × 2 replications, 2014 – 1 sample × 2 replications). Additionally, above ground part of a plant of each genotype, sampled in 2014 at flowering, was fractionated into morphological plant parts: stems, leaves and flowers (3 samples × 2 replications). The samples were washed thoroughly with tap water, rinsed with distilled water and blotted on filter paper. Then they were chopped, fixed at 105°C for 15 min, oven-dried at 65 ± 5°C and ground to

isoflavones in Lithuanian red clover cultivars had not been investigated before either.

The objective of this study was to quantify the concentrations of isoflavones in the cultivars and ecotypes of red clover in a whole above ground part of a plant and separately in stems, leaves and flowers at flowering stage.

Materials and methods

Plant material and trial conditions. Experiments were conducted in the Central Lowland of Lithuania (55°23'49" N, 23°51'40" E), at the Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry. The soil of the experimental site is *Endocalcari-Epihypogleyic Cambisol (CMg-p-w-can)*. Eight cultivars and three wild ecotypes of red clover (*Trifolium pratense* L.) were tested in the germplasm collection (Table).

pass a 1-mm screen. The samples were processed on the same day they were harvested. A small portion (2–3 g) of each sample was dried to a constant mass in an forced-air oven at 105 ± 5°C. The difference in mass before and after the drying process was used to determine the dry matter content.

Extraction of isoflavones. The extraction was performed according to a slightly modified procedure described by Saviranta et al. (2008). The representative amount of sample (0.250 g) was weighed into a 20 mL glass flask with a screw cap and 10 ml of methanol/water (8:2, v/v) containing 2 mol l⁻¹ HCl was added. The mixture was sonicated for 30 min at room temperature and then incubated in a water bath at 80–85°C for 1.5 h with magnetic stirring. The extract was filtered through a 0.2 µm nylon syringe filter and analysed. Before ultra performance liquid chromatographic (UPLC) analysis, leaf extracts of red clover genotypes were diluted with aqueous methanol (1:1, v/v).

Chromatographic conditions. Chromatographic separations were carried out using a Waters Acquity UPLC system (“Waters”, USA) equipped with a binary pump, membrane degasser, autosampler, thermostated column compartment and a photodiode array (PDA) detector. Acquity UPLC BEH C18 column (100 mm column length, 2.1 mm inner diameter, 1.7 µm particle size) was used in the experiments. The column temperature was maintained at 30°C. The injection volume was 5 µl and the injection was performed in a partial loop with a needle overfill injection mode. Elution was performed using 0.25% aqueous acetic acid (mobile phase A) and

0.25% acetic acid in 80:20 v/v methanol/water (mobile phase B). The flow rate was 0.25 ml min⁻¹ with a linear gradient from 2% to 100% B in 15 min, followed by a reequilibration with an initial mobile phase of 5 min. The detection wavelength was set at 260 nm. Data collection and management were performed by software *HyStar 3.2* (“Bruker”, USA).

Identification and quantification. Isoflavones in the extracts were identified according to our recently published procedure (Taujenis et al., 2015). Quantification was performed by external calibration. Isoflavone standards were prepared covering a concentration range from up to 100 mg l⁻¹ with seven levels and three replicates at each level. A good linear relationship between the peak area and concentration was obtained for each of the four isoflavones over the tested concentration range with correlation coefficients $R \geq 0.9989$. The limits of quantification (LOQ), defined as the concentration resulting in a signal of ten times the noise level, were 0.15 mg l⁻¹ (0.006 mg g⁻¹) for biochanin A and formononetin, 0.20 mg l⁻¹ (0.008 mg g⁻¹) for genistein and 0.25 mg l⁻¹ (0.010 mg g⁻¹) for daidzein. Isoflavone concentrations were expressed on a dry matter basis. Results reported are the means \pm standard error (SE) (mg g⁻¹ on DM basis) of three replicate determinations.

The statistical analysis was done using statistical packages *STAT* and *ANOVA* adapted in the *Visual Basic* for application as macro program to run in the *Excel* (Tarakanovas, Raudonius, 2003). Pearson’s correlation coefficients were determined among isoflavone concentration and genotype ploidy level as well as isoflavone concentration and dry matter yield.

Results and discussion

Isoflavones concentration mg g⁻¹ DM at flowering stage. Total isoflavone (formononetin + biochanin A + daidzein + genistein) concentration of 8 cultivars and 3 wild ecotypes was different (Fig. 1). The analysed genotypes formed the following sequence according to the total isoflavone concentration in mg g⁻¹ DM: ‘Radviliai’ (8.09) > ‘Vyliai’ (7.29) > No. 2739 (7.15) > No. 2331 (6.92) > ‘Kiršiniai’ (6.90) > No. 2177 (6.47) > ‘Kamaniai’ (6.41) > ‘Arimaičiai’ (6.28) > ‘Sadūnai’ (6.17) > ‘Vyčiai’ (6.11) > ‘Liepsna’ (5.44).

Diploid cv. ‘Radviliai’, tetraploid cv. ‘Vyliai’ and semi-natural ecotype No. 2739 accumulated significantly ($P < 0.01$) the highest concentration of isoflavones. Significantly the lowest ($P < 0.01$) isoflavone concentrations at flowering stage were accumulated by an old cv. ‘Liepsna’, diploid commercial cv. ‘Vyčiai’ and tetraploid commercial cv. ‘Sadūnai’. Isoflavone concentrations accumulated by diploid cultivars – a commercial cv. ‘Arimaičiai’ and an old cv. ‘Kamaniai’, tetraploid cv. ‘Kiršiniai’ and wild ecotypes Nos. 2177 and 2331 – were average.

Tetraploid cultivars as well as commercial diploid cultivars of red clover mostly had advantage by dry matter yield over wild ecotypes and old diploid cultivars which originated from the natural environment (Table). But supposedly, that wild ecotypes living in natural environment and forming up insignificant yield may provide advantage over modern cultivars by some quality traits in our particular case – increased concentration of secondary metabolites (isoflavones). We measured phenotypic Pearson correlation coefficients between

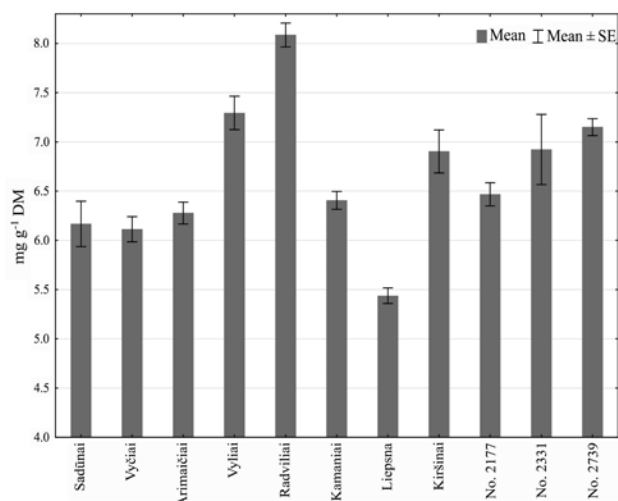


Figure 1. The total isoflavone (formononetin + biochanin A + daidzein + genistein) concentration of red clover genotypes at flowering stage (2013–2014)

isoflavone concentration and genotype ploidy level as well as the relationship between isoflavone concentration and dry matter yield in new commercial cultivars and wild ecotypes as well as old cultivars. Results showed that ploidy level and dry matter yield had no statistically significant effect on isoflavone concentration ($r = 0.355$, $P = 0.28$ and $r = 0.228$, $P = 0.49$, respectively). Partly our findings (average isoflavones concentrations of wild ecotypes Nos. 2177 and 2331) can be explained by the idea presented in the previous study that the natural stressors on the plants are removed and, therefore, the concentration of secondary metabolites are greatly reduced when plants of wild origin are cultivated under “optimal” growing conditions (Gorelick, Bernstein, 2014).

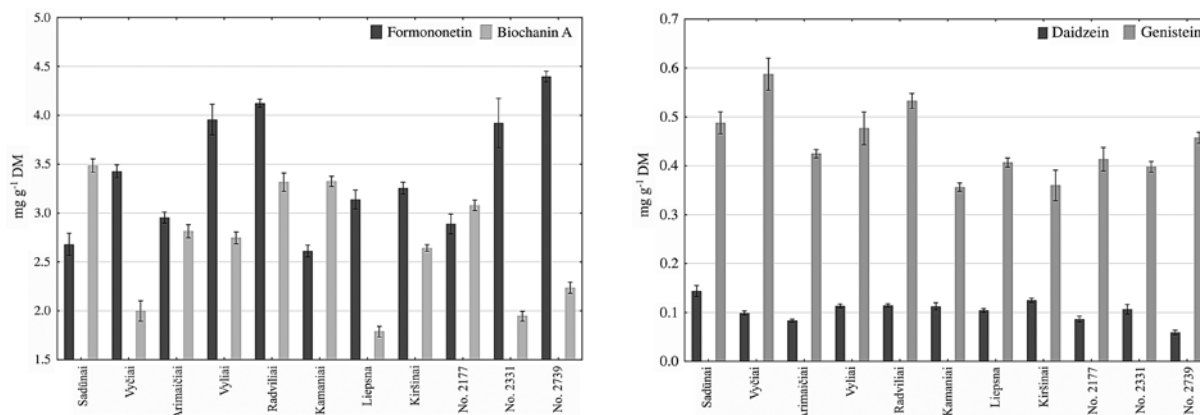
It can be observed that red clover accumulated the highest concentration of formononetin (51%) and biochanin A (40%) at flowering stage (Fig. 2). Daidzein and genistein represented only a small proportion (2% and 7%) of the total isoflavone concentration.



Figure 2. Proportion (%) of formononetin, biochanin A, daidzein and genistein of all red clover genotypes at flowering stage (2013–2014)

Concentrations of formononetin, biochanin A, daidzein and genistein at flowering stage ranged between 2.61–4.40, 1.79–3.32, 0.06–0.14 and 0.36–0.59 mg g⁻¹ DM, respectively (Fig. 3).

The average formononetin concentration of 11 genotypes was 3.41 mg g⁻¹ DM, that of biochanin A – 2.70, daidzein – 0.10 and genistein – 0.45 mg g⁻¹ DM. The average concentration of all four isoflavones at flowering stage during two years of investigations was



Note. Error bars indicate standard error (SE).

Figure 3. Isoflavone concentrations of red clover genotypes at flowering stage (2013–2014)

6.66 mg g⁻¹ DM and the variations ranged from 5.4 to 8.09 mg g⁻¹ DM (Fig. 1).

Isoflavones in the above ground part of red clover have been investigated in detail by other researchers (Rijke de et al., 2004; Sivesidin, Seguin, 2005; Tsao et al., 2006; Oleszek et al., 2007; Ramos et al., 2008; Saviranta et al., 2008). In all these works it was estimated that two isoflavones – formononetin and biochanin A – are dominant in plant material of red clover.

A semi-natural ecotype No. 2739 and diploid cv. ‘Radviliai’ accumulated significantly ($P < 0.01$) the highest concentration of formononetin, 4.40 and 4.12 mg g⁻¹ DM, respectively; significantly ($P < 0.01$) the highest concentration of biochanin A – tetraploid cv. ‘Sadūnai’, diploid cvs. ‘Kamaniai’ and ‘Radviliai’ – 3.49, 3.35 and 3.34 mg g⁻¹ DM, respectively. Tetraploid cvs. ‘Sadūnai’ and ‘Kiršiniai’ accumulated significantly ($P < 0.01$) more daidzein (0.13 and 0.11 mg g⁻¹ DM) compared with other

genotypes, and diploid cvs. ‘Vyčiai’ and ‘Radviliai’ accumulated significantly ($P < 0.01$) more genistein (0.54 and 0.52 mg g⁻¹ DM).

Isoflavone concentration mg g⁻¹ DM in leaves, stems and flowers at flowering stage. The highest concentration of total isoflavones (formononetin + biochanin A + daidzein + genistein) at flowering stage was accumulated by leaves (Fig. 4). Formononetin, biochanin A, daidzein and genistein concentrations in leaves at flowering stage ranged between 3.98–7.17, 4.57–6.86, 0.06–0.17 and 0.49–0.72 mg g⁻¹ DM, respectively. Average concentration of formononetin in leaves of 11 genotypes was 5.77 mg g⁻¹ DM, that of biochanin A – 5.76 mg g⁻¹ DM, daidzein – 0.11 mg g⁻¹ DM and genistein – 0.64 mg g⁻¹ DM. Average concentration of all isoflavones in the leaves of all 11 genotypes was 12.29 mg g⁻¹ DM, and ranged from 9.40 to 14.37 mg g⁻¹ DM.

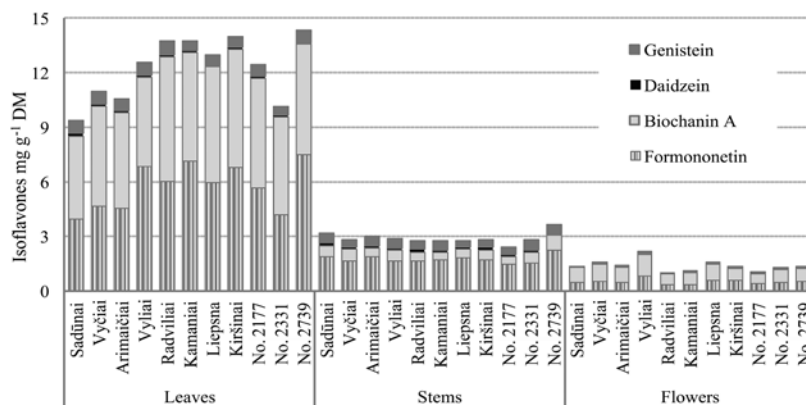


Figure 4. The total isoflavone (formononetin + biochanin A + daidzein + genistein) concentration in the morphological fractions of red clover genotypes (2014)

Formononetin, biochanin A, daidzein and genistein concentrations in stems at flowering stage ranged between 1.49–2.25, 0.44–0.86, 0.06–0.22 and 0.37–0.56 mg g⁻¹ DM, respectively. Average concentration of formononetin in 11 genotypes was 1.76 mg g⁻¹ DM, that of biochanin A – 0.56 mg g⁻¹ DM, daidzein – 0.13 mg g⁻¹ DM and genistein – 0.48 mg g⁻¹ DM. Average concentration of all isoflavones in stems of all 11 genotypes was 2.93 mg g⁻¹ DM and ranged from 2.43 to 3.66 mg g⁻¹ DM.

Formononetin, biochanin A, daidzein and genistein concentrations in flowers at flowering stage ranged between 0.36–0.84, 0.57–1.16, 0.001–0.01 and

0.05–0.23 mg g⁻¹ DM, respectively. Average concentration of formononetin in 11 genotypes was 0.52, biochanin A – 0.78, daidzein – 0.001, genistein – 0.11 mg g⁻¹ DM. Average concentration of all isoflavones in the flowers of all 11 genotypes was 1.41 mg g⁻¹ DM and ranged from 1.09 to 2.23 mg g⁻¹ DM.

Average total concentration of all four isoflavones in a whole aerial plant part at flowering stage was 16.63 mg g⁻¹ DM, the highest one in leaves – 12.29 mg g⁻¹ DM on average, and the lowest one was in flowers – 1.42 mg g⁻¹ DM on average (Fig. 4). Finnish researchers (Saviranta et al., 2008) established the following concentrations of four isoflavones in four

different parts of red clover: 17.56 mg g⁻¹ DM in leaves (the highest value) and 0.54 mg g⁻¹ DM in flowers (the lowest value).

The same trend of total isoflavone concentrations as indicated in literature is clearly observed in the genotypes investigated by us: the highest concentration was established in leaves (9.40–14.37 mg g⁻¹ DM), somewhat lower in stems (2.43–3.66 mg g⁻¹ DM), and the lowest one was recorded in flowers (1.00–2.23 mg g⁻¹ DM). In our investigations, the numerical isoflavone values in morphological fractions of red clover varied marginally. This fact could be related to the specific climatic regional conditions, sampling time, singularities of genotypes and finally – to the differences in the analytical equipment used.

It should be noted that the concentrations of phytoestrogens investigated in red clover plants and their fractions, except flowers, are greater than the total concentration of isoflavones in soybeans. The total concentration of isoflavones in soybeans, which are currently widely used as a source of isoflavones in production of food supplements range from 0.360 to 2.24 mg g⁻¹ (Seguin et al., 2004).

T. pratense is a highly promising source of phytoestrogens for dietary supplement production. All the tested cultivars and wild ecotypes of red clover could be used in the pharmaceutical industry as a source of isoflavones. Especially valuable genotypes were commercial diploid cv. ‘Radviliai’, tetraploid cv. ‘Vyliai’ and semi-natural ecotype No. 2739, which contained the highest concentrations of isoflavones in their leaves.

Conclusions

1. Red clover (*Trifolium pratense* L.) accumulated the highest concentrations of formononetin (51%) and biochanin A (40%) at flowering stage. The concentrations of formononetin, biochanin A, daidzein and genistein at flowering stage ranged between 2.61–4.40, 1.79–3.32, 0.06–0.14 and 0.36–0.59 mg g⁻¹ dry matter (DM), respectively. The average formononetin concentration of 11 genotypes was 3.02, that of biochanin A – 2.70, daidzein – 0.10 and genistein – 0.45 mg g⁻¹ DM. The average concentration of all four isoflavones at flowering stage during the two experimental years was 6.66 mg g⁻¹ DM and ranged from 5.4 to 8.09 mg g⁻¹ DM.

2. The genotypes analysed formed the following sequence according to the total concentration of isoflavones mg g⁻¹ DM: ‘Radviliai’ (8.09) > ‘Vyliai’ (7.29) > No. 2739 (7.15) > No. 2331 (6.92) > ‘Kiršiniai’ (6.90) > No. 2177 (6.47) > ‘Kamaniai’ (6.41) > ‘Arimaičiai’ (6.28) > ‘Sadūnai’ (6.17) > ‘Vyčiai’ (6.11) > ‘Liepsna’ (5.44).

3. The highest concentration of isoflavones at flowering stage was accumulated in leaves. The average total concentration of all four isoflavones at flowering stage was as follows: 12.29 mg g⁻¹ DM in leaves, 2.93 mg g⁻¹ DM in stems and 1.42 mg g⁻¹ DM in flowers.

4. Plant material of all the tested cultivars and wild ecotypes, especially leaves of the commercial diploid cultivar ‘Radviliai’, tetraploid cultivar ‘Vyliai’ and semi-natural ecotype No. 2739, containing the highest concentrations of isoflavones, could be used in the pharmaceutical industry as a source of isoflavones for dietary supplement production.

5. Ploidy level and yield had no statistically significant impact on isoflavone concentration.

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Izoflavonų koncentracija raudonojo dobilo (*Trifolium pratense* L.) žydėjimo metu

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Santrauka

Siekta nustatyti raudonojo dobilo veislių bei ekotipų izoflavonų kiekį žydėjimo tarpsniu visoje augalo antžeminėje dalyje ir atskirai stiebuose, lapuose bei žieduose. Izoflavonai buvo ekstrahuoti parūgštintu vandens bei metanolio tirpalu ir nustatyti ultraefektyviosios skysčių chromatografijos su fotodiodiniu detektoriumi metodu.

Žydėjimo tarpsniu daugiausia formononetino (51 %) ir biochanino A (40 %) sukaupė raudonieji dobilai: formononetino, biochanino A, daidzeino ir genisteino koncentracijos buvo atitinkamai 2,61–4,40, 1,79–3,32, 0,06–0,14 ir 0,36–0,59 mg g⁻¹ sausųjų medžiagų (SM). Visų keturių izoflavonų vidutinė koncentracijų suma per dvejus tyrimo metus žydėjimo tarpsniu buvo 6,66 mg g⁻¹ SM ir svyravo nuo 5,4 iki 8,09 mg g⁻¹ SM. Tirti genotipai pagal bendrą visų tirtų izoflavonų koncentraciją pasiskirstė taip: ‘Radviliai’ (8,09) > ‘Vyliai’ (7,29) > Nr. 2739 (7,15) > Nr. 2331 (6,92) > ‘Kiršiniai’ (6,90), > Nr. 2177 (6,47) > ‘Kamaniai’ > (6,41) > ‘Arimaičiai’ (6,28) > ‘Sadūnai’ (6,17) > ‘Vyčiai’ (6,11) > ‘Liesna’ (5,44) mg g⁻¹ SM. Žydėjimo tarpsniu daugiausia izoflavonų sukaupė lapai. Visų keturių izoflavonų vidutinė koncentracijų suma lapuose buvo 12,29, stiebuose – 2,93, žieduose – 1,42 mg g⁻¹ SM. Visų tirtų veislių ir laukinių ekotipų žaliava, ypač turinti didžiausią koncentraciją izoflavonų – komercinės diploidinės veislės ‘Radviliai’, tetraploidinės veislės ‘Vyliai’ ir pusiau laukinio ekotipo Nr. 2739 lapai – galėtų būti naudojama farmacijos pramonėje kaip izoflavonų šaltinis.

Reikšminiai žodžiai: antžeminė dalis, bioaktyvūs junginiai, biochaninas A, daidzeinas, formononetinas, genisteinas.