

# Source of variation of isoflavone concentrations in perennial clover species

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Submitted: 01-10-2012

Revised: 11-11-2012

Published: 21-02-2014

## ABSTRACT

**Introduction:** Clover has attracted considerable interest not only as a valuable livestock forage plant, but also as an important source of isoflavones. The current study was aimed to assess the variation of concentration of three isoflavones in clover species grown under a cool temperate climate environment in Lithuania. **Materials and Methods:** Isoflavone contents were quantified in the plant parts of 21 accessions belonging to five perennial species of genus *Trifolium* (*T. pratense*, *T. repens*, *T. medium*, *T. rubens*, and *T. pannonicum*). Daidzein, formononetin, and genistein concentrations were quantified in separate plant parts by reversed-phase high-performance liquid chromatography. The analyte extraction was performed from dried and ground leaves, stems, flowers, and roots. The procedure included acid hydrolysis of isoflavone glycosides to aglycones. **Results:** According to the averaged sum of the three isoflavones quantified in leaves-stems-flowers, the five clover species ranked as follows: *T. medium* (7.54-3.62-2.31 mg/g) > *T. pratense* > *T. rubens* > *T. pannonicum* > *T. repens* (0.191-0.204-0.171 mg/g). The contribution of individual compound to the total isoflavone content depended on the species, accession, and plant part. The major part of the isoflavones is concentrated in leaves or stems; however, there is a great variation also. **Conclusion:** There exists a large variation in the total as well as in individual concentration of isoflavones among the clover species and plant parts and within species. With regard to isoflavone concentration and variability within species, some accessions of *T. medium* and *T. pratense* can be considered a highly promising source of phytoestrogens.

**Key words:** Concentration variation, daidzein, formononetin, genistein, plant part, *Trifolium* species and accessions

## INTRODUCTION

The *Trifolium* genus (Fabaceae) comprises about 300 species, found chiefly in north temperate regions, but also, like other north temperate genera, on the mountains in the tropics.<sup>[1,2]</sup> Traditionally, clover is used as a livestock forage and is highly valued due to its ability to fix atmospheric nitrogen in the soil. Moreover, it could be used to meet specific agricultural and environmental needs: For the conservation of eroded or agriculturally disadvantaged soils,<sup>[3]</sup> for planting on dry hill slopes,<sup>[1]</sup> and on acid, saline, dry soils, as well as by using it in apiculture because of the high nectar content

etc.<sup>[4]</sup> Due to the highly ornamental flowers, the plants of *T. fragiferum* L., *T. pannonicum* Jacq. *T. rubens* L. can be used for the establishment of flowering meadows.<sup>[5]</sup>

Isoflavones are a subgroup of phytoestrogens, natural plant substances with a structure similar to 17- $\beta$ -estradiol and capable of binding to estrogen receptors.<sup>[6]</sup> Thanks to the phytoestrogenic effect, isoflavones can prevent or slow up some forms of hormone-dependent cancers, osteoporosis, Alzheimer, cardiovascular pathologies, and relieve symptoms of menopause.<sup>[6-9]</sup> Different isoflavones are specific to fabaceous plants. Many isoflavone products derived from fabaceous are currently widely used as phytoestrogen food supplements (iseren, estro-cal, aterokleft, mabelle, and meflovon, etc.). At present, soybean is the most important isoflavone source. However, its use is limited because of the allergenic reactions it can cause in some people.

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### Access this article online

#### Website:

www.phcog.com

#### DOI:

10.4103/0973-1296.127373

#### Quick Response Code:



Moreover, production area of genetically modified soy is steadily increasing and many people do not approve of the use of such plants.<sup>[10]</sup> Therefore, scientists are seeking for new isoflavone resources. Since olden times, red clover (*T. pratense* L.) has been used in folk medicine for treatment of some diseases (eczemas, asthma, and eye conditions, etc.).<sup>[11]</sup> About 40 different isoflavones are detected in red clover,<sup>[12,13]</sup> of which the most common are formononetin, biochanin A, daidzein, and genistein<sup>[14,15]</sup> and the last two compounds are the primary isoflavones in soy. Research related to isoflavone distribution in clover is predominantly limited to one species—red clover.<sup>[16-18]</sup> Little evidence is available on isoflavones in other *Trifolium* species and their variation within and between species. The current study focuses on the aspects of variation of isoflavone concentration in five clover species grown under a cool temperate climate environment in Lithuania.

## MATERIALS AND METHODS

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 during 2007–2008. The soil of the experimental site is *Endocalcari-Epiphypogleyic Cambisol (CMg-p-w-can)*. The isoflavone studies involved germplasm collection of the following perennial clover species: *T. medium* L., *T. pannonicum* Jacq., *T. pratense* L., *T. repens* L., and *T. rubens* L. The collection included commercial varieties and wild ecotypes, collected in natural or seminatural habitats in Lithuania and the Ukraine, and seeds of one wild population of *T. pannonicum* were provided by the French colleagues, the seed origin was not indicated [for details see Table 1].

Two samples (one wild ecotype of *T. medium* no. 2148 with a high isoflavone content and one variety of *T. repens* “Sūduviai” characterized by a negligible isoflavone concentration) were tested for these compounds in the first (1) and second (2) years of their cultivation. In 2007 and 2008, genetic collections of clover species were established in a trial field using seedlings grown in a greenhouse. Thirty plants per accession (2 replications, 15 plants per replication) were planted at 50 × 50 cm distances. The clover plants were cut at full flowering stage, aerial part of plants was separated into three fractions: Stems, leaves together with petioles, and flowers. Fresh samples of each fraction were immediately chopped into particles of 3-5 cm, fixed at 105°C for 20 min, dried at (65 ± 5) °C and ground in a cyclonic mill with a 1 mm sieve. Dried samples were assayed for the following isoflavones: Daidzein, formononetin, and genistein.

### Chemicals

An HPLC grade acetonitrile and trifluoroacetic acid

were purchased from Sigma-Aldrich (Steinheim, Germany), ethanol from Stumbras AB (Kaunas, Lithuania). Ultrapure water (>18 MΩ cm) was used throughout the HPLC experiment. Standards of daidzein or 4', 7- dihydroxyisoflavone (purity 99.9%) and genistein also known as 4', 5, 7-trihydroxyisoflavone (purity 99.3%) were obtained from ChromaDex (Irvine, CA, USA) and formononetin (7-hydroxy-4'-methoxyisoflavone) (purity ≥ 99%) from Fluka-Sigma-Aldrich (Steinheim, Germany). The standard solutions were prepared by dissolving standard compounds in methanol (0.2-30 µg/mL).

### HPLC equipment and isoflavone quantification

The sample preparation procedure included acid hydrolysis of glycoside forms and was performed under slightly modified conditions described in the USP monograph.<sup>[19]</sup> The isoflavones were extracted with 60 mL of 50% v/v ethanol from 1500 mg of accurately weighed ground plant material (orbital shaker, 12 h). After extraction, the solution was filtered. A volume of 50 mL of the filtrate was transferred to a round-bottom flask and evaporated to dryness under reduced pressure. The residue was rinsed with 10 mL of 96.3% v/v of ethanol and 10 mL of water and 5 mL of 10 M HCl was added into the flask and the mix was heated for 30 min on the water bath using the reflux condenser. The filtered hydrolyzate was mixed with 50% v/v of ethanol to bring the volume up to 30 mL and final solution was filtered

**Table 1: List of germplasm collection of the *Trifolium* spp. examined for isoflavones**

Species	Accession	Notation	Origin (year of use)
<i>T. medium</i>	2148	Tme 1	Wild ecotype, Lithuania (1)
	2148	Tme 2	Wild ecotype, Lithuania (2)
	41	Tme 3	Wild ecotype, Lithuania (1)
	2169	Tme 4	Wild ecotype, Lithuania (1)
	40	Tme 5	Wild ecotype, Lithuania (1)
	101	Tme 6	Wild ecotype, Ukraine (1)
<i>T. pratense</i>	Vyčiai	Tpr 1	Variety, Lithuania (1)
	Ilte	Tpr 2	Variety, Estonia (1)
	Vyliai	Tpr 3	Variety, Lithuania (1)
	Arimaičiai	Tpr 4	Variety, Lithuania (1)
<i>T. repens</i>	Sūduviai	Tre 1	Variety, Lithuania (1)
	Sūduviai	Tre 2	Variety, Lithuania (2)
	Nemuniai	Tre 3	Variety, Lithuania (1)
	Bitūnai	Tre 4	Variety, Lithuania (1)
	1459	Tre 5	Wild ecotype, Lithuania (1)
<i>T. pannonicum</i>	107	Tpa 1	Wild ecotype, Ukraine (1)
	105	Tpa 2	Wild ecotype, Ukraine (1)
	2248-G	Tpa 3	Wild ecotype, unknown (France?) (1)
<i>T. rubens</i>	108	Tru 1	Wild ecotype, Lithuania (1)
	2438	Tru 2	Wild ecotype, Ukraine (1)
	UA	Tru 3	Wild ecotype, Ukraine (1)

through a nylon membrane having a 0.45  $\mu\text{m}$  porosity (Millipore, MA, USA). The analyses were carried out with a Waters 2695 Alliance HPLC system (Milford, MA, USA), equipped with a Waters 2487 UV/Vis detector and a Waters XTerra RP18 150  $\times$  3.9 mm column with a guard column. The dual  $\lambda$  absorbance detector allowed monitoring of two wavelengths, 258 nm (for genistein analyte) and 301 nm (for daidzein and formononetin analytes). Waters 996 photodiode array detector (PDA) detector was used for peak spectral identification. Chromatographic and PDA data were recorded and peak area responses were integrated by the software Waters Empower2 (Milford, MA, USA). The gradient chromatographic elution was carried out according to Raudonis *et al.*,<sup>[20]</sup> The linear through zero type calibration curves were generated using external standard solutions, prepared by dissolving standard compounds in methanol (0.2-30  $\mu\text{g}/\text{mL}$ ). The dependence between peak area and concentration was linear with the determination coefficient  $R^2 \geq 0.9998$  for each of the three isoflavone. The regression equations for the standard curve of genistein, daidzein, and formononetin were respectively:  $y = 160329691 \cdot x$ ,  $y = 55761134 \cdot x$ , and  $y = 55059861 \cdot x$  ( $y$  refers to the peak area,  $x$  - to the concentration of the respective reference). Limit of detection was determined with standard solution at a signal-to-noise ratio of three and was  $< 0.078 \mu\text{g}/\text{mL}$  (for daidzein),  $< 0.032 \mu\text{g}/\text{mL}$  (for genistein), and  $< 0.072 \mu\text{g}/\text{mL}$  (for formononetin). Isoflavone concentrations were expressed as mg/g of sample dry matter (DM).

## RESULTS AND DISCUSSION

It is commonly known that red clover is a rich source of isoflavones, which have been extracted and commercialized as phytoestrogen products or nutraceuticals Rimostil,

Promensil from Novogen, Australia,<sup>[21,22]</sup> Menoflavan<sup>®</sup> from Melbrosin, Austria.<sup>[23]</sup> Our experimental evidence suggests that this species is quite abundant in isoflavones (formononetin + daidzein + genistein): Averaged total concentration depending on an aerial plant part amounted to 2.22 (flowers)-3.32 (stems) mg/g [Table 2]. The total concentration of these three isoflavones of similar order in *T. pratense* (3.25 mg/g) was observed also by Zgórka.<sup>[24]</sup> Such concentration of the three phytoestrogens tested (excluded biochanin A) in red clover is equal or even higher than the total isoflavone concentration in soy: In soybean it ranges from 0.360 to 2.24 mg/g.<sup>[25]</sup>

Although *T. pratense* has received a lot of researchers' interest, the highest isoflavone contents are accumulated by the aerial plant parts of perennial *T. medium*. During flowering stage, the leaves of the *T. medium* contained, on average, the highest total concentration (7.54 mg/g) of the three isoflavones. However, depending on an accession, this concentration varied within a very wide range from 1.45 to 15.2 mg/g with the standard deviation equal to 5.52. The average total isoflavone concentration in stems of this species was 3.62 mg/g and it varied in a narrower range than in leaves: From 1.54 to 4.87 mg/g. The lowest average isoflavone concentration (2.307 mg/g) was found in flowers of *T. medium* and ranged from 0 to 5.07 mg/g. Only negligible concentrations of the compounds assayed were determined in all aerial plant parts of *T. repens*, *T. pannonicum*, and *T. rubens*. Screening of 57 *Trifolium* species for total isoflavone concentration, done by Oleszek *et al.*,<sup>[26]</sup> showed also that there are a number of species with particularly high amounts of these compounds. However, the regularity of isoflavone content variation between species significantly differed from that established in our study as well as Zgórka<sup>[24]</sup> research: According to Oleszek *et al.*,<sup>[26]</sup> isoflavone concentration in *T. pratense* ranged from 10.39 to 39.51, and in *T. medium*

**Table 2: Variation of the total isoflavone concentration among aerial plant parts and clover species**

Aerial plant part	Parameter name	<i>Trifolium</i> spp.				
		<i>T. medium</i>	<i>T. pratense</i>	<i>T. repens</i>	<i>T. pannonicum</i>	<i>T. rubens</i>
Stems	AVG	3.62	3.32	0.204	0.230	0.780
	Max	4.87	4.74	0.279	0.466	1.63
	Min	1.54	1.52	0.086	0.019	0.033
	SD	1.32	1.34	0.072	0.225	0.804
Leaves	AVG	7.54	2.74	0.191	0.274	0.493
	Max	15.19	4.29	0.260	0.736	1.43
	Min	1.45	1.11	0.136	0.030	0.011
	SD	5.52	1.39	0.047	0.400	0.812
Flowers	AVG	2.31	2.22	0.171	0.980	0.380
	Max	5.07	4.17	0.293	2.36	0.948
	Min	0.012	0.990	0.098	0.023	0.039
	SD	1.72	1.36	0.073	1.23	0.495

Note: Data with isoflavone concentration  $< \text{LOD}$  were included for average (AVG) and standard deviation (SD) calculation

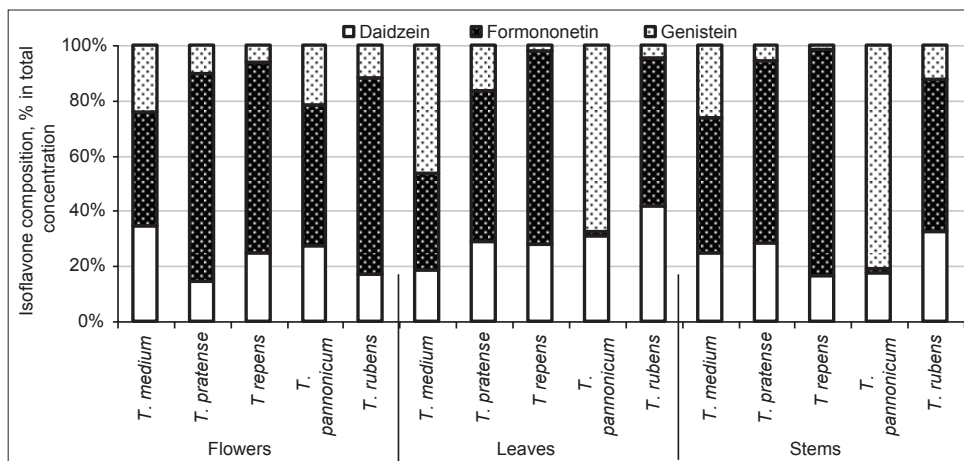
it was as low as 1.58 mg/g DM. Considering the average isoflavone content, our findings in general are consistent with the regularity recently established by Zgórka<sup>[24]</sup>: Lower isoflavone contents have been determined for white and white-pink flowering clover species (*T. repens* and *T. pannonicum*) and in clover species, whose flowers are characterized by red color, isoflavone content was higher, except for *T. rubens*. Dark red color is specific to *T. rubens* flowers and the isoflavone contents detected varied within 0.011-1.63 mg/g range [Table 2]. There is considerable variation in total and individual isoflavone concentrations between the plant parts. Research sources provide contradictory findings on which above-ground plant parts accumulate maximal isoflavone contents.<sup>[17,27]</sup> According to Vetter,<sup>[16]</sup> isoflavone accumulation in different plant organs is species-specific. In our study, for *T. pratense*, *T. rubens*, and *T. repens* higher isoflavone concentrations were detected in stems, while for *T. pannonicum* in flowers. Leaves of *T. medium* were richer in isoflavones than any other above-ground plant part.

Differences between specific isoflavones also varied greatly depending on species and plant part [Figure 1]. From some literature sources, it follows that formononetin like biochanin A (not analyzed in our work) concentration in whole plant and/or individual plant parts is from twice to much more times higher than that of daidzein and genistein.<sup>[18,24,28-30]</sup> In our study, in most cases formononetin accounted for more than half of the total concentration of the three isoflavones [Figure 1]. In average for *T. pratense* accessions, formononetin content ranged from 54.9% (in leaves) to 74.95% (in flowers). More even distribution of isoflavones was determined in aerial parts of *T. medium* plants, with prevalence of genistein in leaves (46.6%) and formononetin in flowers and stems (40.9% and 49.3%, respectively). The average composition of the three isoflavones in leaves and stems of *T. pannonicum*

species was exceptional: Genistein was a predominant isoflavone in leaves (65.0%) and stems (80.9%), while the concentration of formononetin in these aerial plant parts was only negligible. However, its share in flowers accounted for 51.3% of the total content of the tested compounds.

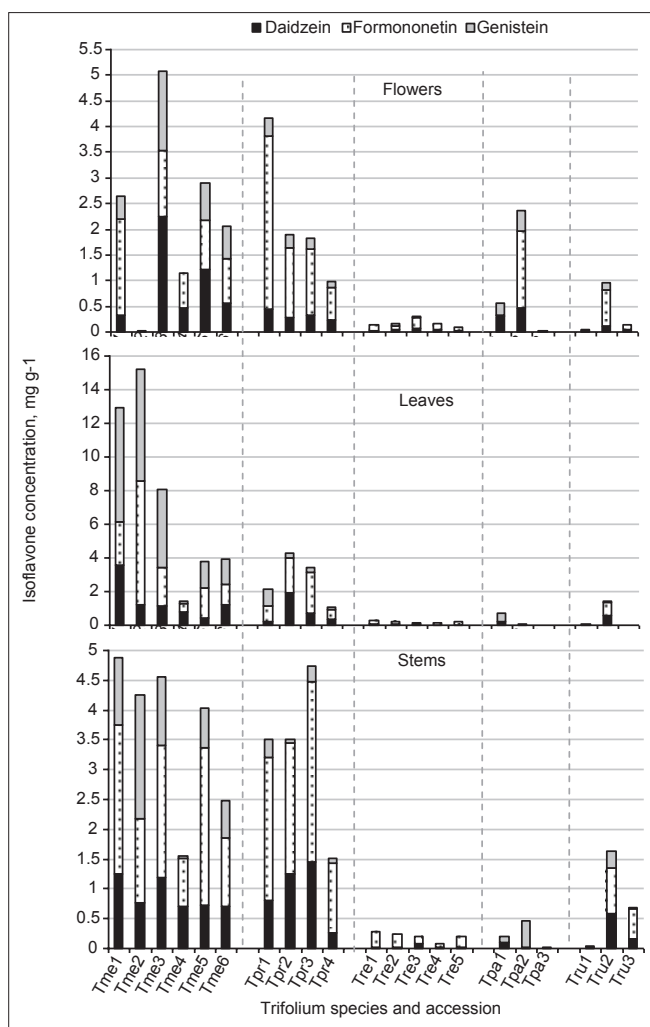
The concentration as well as composition of isoflavones in different accessions within a clover species may differ significantly [Figure 2]. We will pay more attention to intraspecific variation of this attribute in aerial plant parts of the two species, containing high average isoflavone concentration, that is, *T. medium* and *T. pratense*. The concentrations of the isoflavones in six samples of zigzag clover *T. medium* [Tme1-Tme6 in Figure 2] largely varied depending on an accession, plant part, and year of use. Nearly, all populations tested were characterized by a higher total isoflavone concentration in leaves than in other aerial plant parts. The local wild population of *T. medium* no. 2148 was distinguished by the abundance of these compounds in leaves in both years of use (12.9 and 15.2 mg/g in leaves of Tme1 and Tme2, respectively). The flowers of this population accumulated lower concentrations of these bioactive compounds: 2.64 mg/g in the flowers of plants in the first year of use and only trace levels in the second year of use. The leaves, like other aerial plant parts, of other populations also contained high concentrations of isoflavones: The total concentration of the three isoflavones was equal to or markedly exceeded that in soy, which so far has been the most popular source for the production of isoflavone supplements. The plant parts of the Lithuanian wild population Tme4 contained markedly lower concentrations of these compounds; however, their distribution in leaves-stems-flowers was the most even (1.45-1.54-1.15 mg/g).

Among the four *T. pratense* accessions, it is possible to distinguish only one Lithuanian variety “Arimaičiai” (Tpr4), in whose aerial plant parts the total isoflavone concentration



**Figure 1:** Variation of isoflavone composition among clover species and aerial plant parts





**Figure 2:** Variation of isoflavones among aerial plant parts, between and within two clover species. *Note:* Data with isoflavone concentration < LOD were included

was < 2.00 mg/g: From 0.990 mg/g in flowers to 1.52 mg/g in stems. The distribution of isoflavone contents in the plant parts of other accessions was uneven. Among aerial plant parts, the highest concentrations were established in the flowers of Tpr1 (4.17 mg/g), leaves of Tpr2 (4.29 mg/g), and stems of Tpr3 (4.74 mg/g).

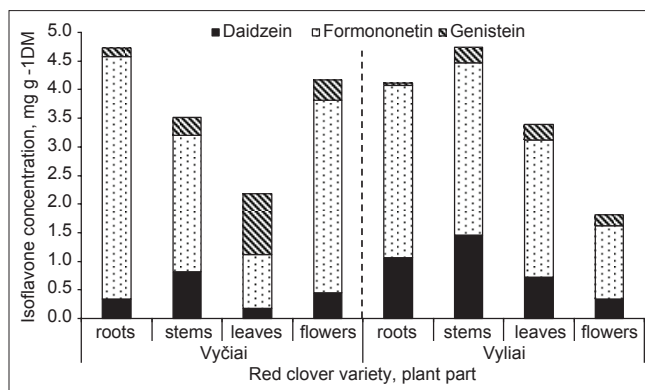
In terms of isoflavone composition, all red clover accessions had formononetin as the predominating isoflavone in stems, flowers, and leaves; formononetin share in the total concentration of the three isoflavones ranged from 42.7% to 80.6%, except for Tpr1 leaves, where genistein accounted for a slightly larger share than formononetin-48.9% or 1.07 mg/g of the total amount 2.18 mg/g [Figure 2]. Similar concentrations of formononetin (2.10 mg/g) and daidzein (1.91 mg/g) were found in the leaves of the red clover variety “Ilte” (Tpr2) of Estonian origin. The major compound of the three isoflavones of some zigzag clovers may not always be formononetin as was

the case with red clovers. The composition of the tested isoflavones in *T. medium* varied inconsistently. In leaf samples of Tme1, Tme3, and stem samples of Tme2, the contribution of genistein to the sum of isoflavones was ultimate among the compound tested: 52.3%, 57.6%, and 48.7%, respectively. Wild ecotype Tme4 was distinguished not only for lower total isoflavone concentration among zigzag clovers, but also for their composition with negligible content of genistein and quite high share of daidzein and formononetin, accounting for 40.2%-53.4% and from 36.2% to 59.4% of the sum of isoflavones in the aerial plant parts [Figure 2]. Isoflavone concentrations in clover, like those of many secondary metabolites, is primarily associated with the genetic peculiarities of species/accession.<sup>[28]</sup> Such differences were obvious not only among the *T. medium* and *T. pratense* accessions. In a flower sample of one of the three *T. pannonicum* populations (Tpa2 from Ukraine), a moderate isoflavone concentration, that is, 2.36 mg/g was determined. Similarly, in *T. rubens* species, the wild ecotype Tru2 of Ukrainian origin too, accumulated in the aerial plant parts from 0.948 (flowers) to 1.63 (stems) mg/g isoflavones, which is two to three times more than in average for the species. Some differences in isoflavone concentration as well as composition may be explained also by different growing conditions (temperature, light, nutrition, etc.),<sup>[30]</sup> but this factor in our study was not relevant. The cause of the fluctuations in isoflavone concentration and composition between years of use could not be defined correctly because only two populations were tested for isoflavones in the first and second years of their cultivation, and exact developmental stages were not recorded. As Sivesind and Seguin<sup>[31]</sup> observed, changes in isoflavone concentrations with increasing maturity varied depending on the plant part: The highest isoflavone concentrations were found in leaves and stems during the vegetative stages, with a decline until plants initiated flowering (principally at the expense of formononetin), and then the concentrations stabilized in both parts. The concentration of isoflavones in flowers decreased more sharply.

In order to further determine the role of plant part in the isoflavones concentration, the Lithuanian varieties of red clover “Vyčiai” and “Vyliai” were additionally analyzed for daidzein, genistein, and formononetin contents in roots [Figure 3]. The data are presented by comparing the distribution of these compounds in aerial plant parts. The roots are characterized by quite high total levels of isoflavones tested; moreover, the below-ground plant part was the most abundant in isoflavones (4.73 mg/g) among all plant parts of the variety “Vyčiai”. Other plant parts of this variety were ranked in the following order: Flowers (4.17 mg/g), stems (3.51 mg/g), and leaves (2.18 mg/g). Completely different isoflavone

distribution per plant was characteristic of the variety “Vyliai”. With regard to the total concentration of phytoestrogens tested, plant parts were arranged in the following sequence: Stems (4.74 mg/g), roots (4.11 mg/g), leaves (3.40 mg/g), and flowers (1.82 mg/g). In terms of formononetin concentration differences in plant parts of “Vyčiai”, roots contained the largest amount of this isoflavone. Daidzein together with genistein represented only a small proportion, that is, 10.5% of the total isoflavone, whereas leaves accumulated considerably more genistein, which accounted for about half of the isoflavone sum. Fairly large amount of daidzein was found in stems of “Vyčiai” (0.813 mg/g in 3.51 mg/g of total), as well as in roots, stems, and leaves of “Vyliai” (1.06, 1.46, and 0.724 mg/g or 25.9%, 30.8%, and 21.3% of the total concentration of three isoflavones, respectively).

With regard to species practical application, a very important aspect is high DM yield, DM distribution among individual plant parts, as good reproductive plant characteristics. Previous research of Vilčinskis and Dabkevičienė<sup>[32,33]</sup> showed that DM yield greatly varied among clover species, and according to the productivity per plant averaged for species, five clovers were ranked in the following order: *T. pannonicum*, *T. pratense*, *T. rubens*, *T. medium*, and *T. repens*. High seed yield (4.43-2.42 g per plant) was noted for *T. pratense*, *T. pannonicum*, *T. repens*, and *T. rubens*. Although only moderately yielding *T. medium* plants exhibited low seed set, due to its specific root system (vegetative propagation by rhizomes), persistence and longevity as well as the highest isoflavone content, this species could be a potential medicinal plant in the countries of temperate climate. Furthermore, *T. medium* plants were characterized by quite high percentage of leaves (39.4%), and as is seen from Table 2, the largest isoflavone concentration was found in this aerial plant part of zigzag clover. Among *Trifolium* spp., the significantly highest stem weight (53.5%) was determined in *T. pratense*.<sup>[33]</sup> Since the most considerable isoflavone concentrations in average for red clover were accumulated in stems, this species trait, as well as high DM and seed yield make *T. pratense* one of the most plausible isoflavone sources under Lithuania’s conditions and under those of cool temperate climate countries. Although *T. pannonicum* plants displayed high seed set and the highest DM yield per plant, there was a wide intraspecific variability in the content of isoflavones, and additional research for accession choice is needed, the same is true for *T. rubens*. All five *T. repens* accessions tested showed very low isoflavone concentrations in all aerial plant parts, thus, in our opinion, this species has no prospects as a source of isoflavones. The intraspecific variability in the total and individual isoflavone concentrations, in addition to the variability in agrobiological traits described previously,<sup>[32,33]</sup> indicates that it would be feasible to select populations for the desirable combination



**Figure 3:** Pattern of isoflavones variation among plant parts of two red clover varieties

of chemical and agrobiological properties through a variety development program. Metabolic pathways of aromatic secondary metabolites, including isoflavones, are particularly complex.<sup>[34]</sup> Genetic peculiarities, environmental factors, agronomic practices, and plant maturity have a noticeable impact on the dynamics of qualitative and quantitative accumulation of these compounds. Therefore, it would be profitable to conduct a more comprehensive evaluation of the germplasm at different stages of maturity for the range of isoflavone, including biochanin A.

## CONCLUSIONS

The present study revealed that total and individual concentrations of formononetin, daidzein, and genistein showed great variation among clover species and identified that the isoflavone content in *T. medium* and *T. pratense* species is much higher than that found in the other clover species; therefore, these two species may be valuable multifunctional plants for different use.

This study also confirmed the large variation in the isoflavone concentration and profile among aerial plant parts. Generally, leaves of the zigzag clover and stems of the red clover plants had the highest total isoflavone concentration among all plant parts. But a marked intraspecific difference was also established both in total isoflavone levels and distribution of individual compounds among plant parts.

The concentration of total and individual isoflavones (formononetin, daidzein, and genistein) in the aerial part of clover species was different for the studied accessions. Isoflavone concentration in leaves of the local wild population *T. medium* no. 2148 both of in the first and second years of use was much higher (12.9 and 15.2 mg/g) than that found in stems and flowers, as well as in the other accessions of *Trifolium* spp. There was established a large variation in the total as well

as in individual concentration of isoflavones within other clover species too. The roots of red clover are characterized by high total levels of isoflavones tested; moreover, roots were the most abundant in isoflavones (4.73 mg/g) among all plant parts of the variety “Vyčiai”.

Screening of wild ecotypes and varieties could be useful to identify germplasm with a high potential of bioactive substances, for both wider cultivation of clovers as medicinal plants and for further breeding programs aimed to develop varieties with the desirable combination of chemical and agrobiological properties. To get a better insight into the factors responsible for optimal isoflavone concentrations in clover plants, a more extensive evaluation of germplasm at different stages of maturity for the range of isoflavones is needed.

## ACKNOWLEDGMENTS

This work was partly supported by the long-term research programs “Genetics and purposeful change of genotypes of agricultural and forest plants” and “Biopotential and quality of plants for multifunctional use” implemented by Lithuanian Research Centre for Agriculture and Forestry.

## REFERENCES

- Gillett JM, Taylor NL, Collins M. The world of clovers. In: Collins M, editor. Iowa State, USA: University Press; 2001. p. 457.
- Clover. The 1911 Classic Encyclopedia. Based on the 11<sup>th</sup> Edition of the Encyclopaedia Britannica (Pub. 1911). Available from: <http://www.1911encyclopedia.org/Clover> [Last accessed on 2012 Sep 5].
- Smith RR. Kura clover (*Trifolium ambiguum* M.B.): A forage legume with great potential for pasture and conservation. In: Boller B, Stadelman FJ, editors. Breeding for a Multifunction Agriculture. Zurich-Reckenholz: Swiss Federal Research Station for Agroecology and Agriculture; 1998. p. 87-90.
- Noor MJ, Khan MA, Camphor ES. Palynological analysis of pollen loads from pollen sources of honeybees in Islamabad, Pakistan. Pak J Bot 2007;41:495-501.
- Rumball W, Claydon RB. ‘Gao 153’ turf type strawberry clover (*Trifolium fragiferum* L. N Z J Agric Res 2005;48:421-2.
- Pilšáková L, Riečanský I, Jagla F. The physiological actions of isoflavone phytoestrogens. Physiol Res 2010;59:651-64.
- Setchell KD. Phytoestrogens: The biochemistry, physiology, and implications for human health of soy isoflavones. Am J Clin Nutr 1998;68(Suppl):1333S-46.
- Ososki AL, Kennelly EJ. Phytoestrogens: A review of the present state of research. Phytother Res 2003;17:845-69.
- Albulescu M, Popovici M. Isoflavones – biochemistry, pharmacology and therapeutic use. Rev Roum Chim 2007;52:537-50.
- Aparicio-Fernandez X, Yousef GG, Loarca-Pina G, de Mejia E, Lila MA. Characterization of polyphenolics in the seed coat of black jamapa bean (*Phaseolus vulgaris* L.). J Agric Food Chem 2005;53:4615-22.
- Wren RC. Potter’s New Cyclopaedia of Botanical Drugs and Preparations. Saffron Walden: C.W. Daniel Company Ltd.; 1988. p. 112.
- Kjeldus B, Vitamvasova D, Kuban V. Identification of isoflavone conjugates in red clover (*Trifolium pratense*) by liquid chromatography-mass spectrometry after two-dimensional solid-phase extraction. Anal Chim Acta 2001;450:81-97.
- Rijke E, Zappey H, Ariese F, Gooijer C, Brinkman UA. Flavonoids in Leguminosae: Analysis of extracts of *T. pratense* L., *T. dubium* L., *T. repens* L., and *L. corniculatus* L. leaves using liquid chromatography with UV, mass spectrometric and fluorescence detection. Anal Bioanal Chem 2004;378:995-1006.
- Liu J, Burdette JE, Xu H, Gu C, van Breemen RB, Bhat KP. Evaluation of estrogenic activity of plant extracts for the potential treatment of menopausal symptoms. J Agric Food Chem 2001;49:2472-9.
- Booth NL, Overk CR, Yao P, Totura S, Deng Y, Hedayat AS. Seasonal variation of red clover (*Trifolium pratense* L., Fabaceae) isoflavones and estrogenic activity. J Agric Food Chem 2006;54:1277-82.
- Vetter J. Isoflavones in different parts of common *Trifolium* species. J Agric Food Chem 1995;43:106-8.
- Lin LZ, He XG, Lindenmaier M, Yang J. LC-ESI-MS study of the flavonoid glycoside malonates of red clover (*Trifolium pratense*). J Agric Food Chem 2000;48:354-65.
- Wu Q, Wang M, Simon JE. Determination of isoflavones in red clover and related species by high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection. J Chromatogr A 2003;1016:195-209.
- Sharaf MH. Red clover. In: Williams RL, editor. US Pharmacopeia 32-NF27. Rockville: United States Pharmacopeia; 2009. p. 983-6.
- Raudonis R, Jakstas V, Burdulis D, Benetis R, Janulis V. Investigation of contribution of individual constituents to antioxidant activity in herbal drugs using post column HPLC method. Medicina 2009;45:382-94.
- Clifton-Bligh PB, Baber RJ, Fulcher GR, Nery ML, Moreton T. The effect of isoflavones extracted from red clover (Rimostil) on lipid and bone metabolism. Menopause 2001;8:259-65.
- Van de Weijer PH, Barentsen R. Isoflavones from red clover (Promensil®) significantly reduce menopausal hot flush symptoms compared with placebo. Maturitas 2002;42:187-93.
- Dornstauder E, Jisa E, Unterrieder I, Krenn L, Kubelka W, Jungbauer A. Estrogenic activity of two standardized red clover extracts (Menoflavon®) intended for large scale use in hormone replacement therapy. J Steroid Biochem Mol Biol 2001;78:67-75.
- Zgórka G. Ultrasound-assisted solid-phase extraction coupled with photodiode-array and fluorescence detection for chemotaxonomy of isoflavone phytoestrogens in *Trifolium* L. (Clover) species. J Sep Sci 2009;32:965-72.
- Seguin P, Zheng W, Smith DL, Deng W. Isoflavone content of soybean cultivars grown in eastern Canada. J Sci Food Agric 2004;84:1327-32.
- Oleszek W, Stochmal A, Janda B. Concentration of isoflavones and other phenolics in the aerial parts of *Trifolium* species. J Agric Food Chem 2007;55:8095-100.
- Seguin P, Zheng W, Souleimanov A. Alfalfa phytoestrogen content: Impact of plant maturity and herbage components. J Agron Crop Sci 2004;190:211-7.
- Tsao R, Papadopoulos Y, Yang R, Young JC, McRae K. Isoflavone profiles of red clovers and their distribution in different parts harvested at different growing stages. J Agric Food Chem 2006;54:5797-805.
- Ramos P, Dias PM, Morais CB, Froehlich PE, Dall’Agnol M, Zuanazzi JA. LC Determination of four isoflavone aglycones

- in red clover (*Trifolium pratense* L.). *Chromatographia* 2008;67:125-9.
30. Saviranta NM, Anttonen MJ, von Wright A, Karjalainen RO. Red clover (*Trifolium pratense* L.) isoflavones: Determination of concentrations by plant stage, flower colour, plant part and cultivar. *J Sci Food Agric* 2008;88:125-32.
  31. Sivesind E, Seguin P. Effects of the environment, cultivar, maturity, and preservation method on red clover isoflavone concentration. *J Agric Food Chem* 2005;53:6397-402.
  32. Vilčinskas E, Dabkevičienė G. Yield structure and dry matter qualitative characteristics of clover (*Trifolium* spp.) species. *Žemės ūkio mokslai* 2010;17:18-24.
  33. Vilčinskas E, Dabkevičienė G. Qualitative and quantitative characteristics of clover (*Trifolium* spp.) species in the first year of growing. *Zemdirbyste-Agriculture* 2009;96:170-80.
  34. Graham TL. A rapid, high resolution high performance liquid chromatography profiling procedure for plant and microbial aromatic secondary metabolites. *Plant Physiol* 1991;95:584-93.

**Cite this article as:** Butkute B, Lemežiene B, Dabkeviciene G, Jakštas V, Vilcinskas E, Janulis V. Source of variation of isoflavone concentrations in perennial clover species. *Phcog Mag* 2014;10:S181-8.

**Source of Support:** Nil, **Conflict of Interest:** None declared.