

Phytoestrogen content in clover (*Trifolium* spp.) and in grass stands depending on treatment and storage

Řepková J.¹, Nedělník J.², Krtková V.³, Schulzová V.³, Novotná H.³, Hajšlová J.³ and Jakešová H.⁴

¹Department of Experimental Biology, Masaryk University Brno, Czech Republic;

²Agricultural Research, Ltd., Troubsko, Czech Republic;

³Department of Food Analysis and Nutrition, Institute of Chemical Technology Prague, Czech Republic;

⁴Red Clover and Grass Breeding, Hladké Životice, Czech Republic

Corresponding author: repkova@sci.muni.cz

Abstract

Beneficial as well as negative effects of phytoestrogens (PEs) on foodstuffs and fodder of plant origin have been studied. In this work, PE levels were examined over 2 years in red clover (*Trifolium pratense*), zigzag clover (*T. medium*) and their hybrid, as well as in haylage from two locations in the Czech Republic produced from the first cutting of grass on perennial grass stands. A statistically significant difference in PE content was found between the two species of clover. Plants in the hybrid population contained a statistically inconclusive difference in PE content in comparison with *T. pratense* and a lesser PE content than *T. medium*. In high- and low-quality haylage samples, a significantly higher PE content was found in samples from the Závěšice location (2011 and 2012 harvest), which may be due to the difference in individual species representations in the fodders at the given locations.

Keywords: phytoestrogens, *Trifolium pratense*, *Trifolium medium*, UHPLC-MS/MS

Introduction

Phytoestrogens (PEs) are biologically active chemical compounds of plant origin which display effects similar to those of oestrogen sex hormones (Velišek *et al.*, 2009). In recent years, interest in these substances has grown with a view to their possibly beneficial but also possibly negative effects on humans and animals. The main and most significant sources of PEs are considered to be pulses (in particular soya beans (*Glycine max*) and fodder crops (red clover (*Trifolium pratense*) and alfalfa (*Medicago sativa*)) which are used as feedstuffs (Kuhnle *et al.*, 2008). The PEs most represented in fodder crops are biochanin A and formononetin while those in pulses are daidzein, genistein and glycitein (Beck *et al.*, 2005). In mammals, PEs are metabolized to products which generally display higher oestrogen activity than those original forms.

The aim of this two-year study was to examine the PE profile of a new hybrid population, which was bred through interspecific hybridisation of red clover and zigzag clover (*T. medium*) (Jakešová *et al.*, 2011). A further aim was to evaluate PE content in high- and low-quality haylage.

Materials and methods

Breeding material acquired by *T. pratense* x *T. medium* interspecific hybridization was tested for content of daidzein, genistein, formononetin and biochanin A in 2011 and 2012. The 'Amos' variety and *T. medium* were parental genotypes used for comparison. The haylages were produced from the first cutting of perennial grass stands at Lukov and Závěšice in the Czech Republic during 2011 and 2012. Haylage was produced and stored in the form of plastic-wrapped hay bales. Low-quality haylage was prepared by artificially damaging the protective plastic film covering the hay bales in several places with the tines of a pitchfork (simulating damage from branches) and by slicing it with a knife (simulating greater damage). Approximately once per week the damaged bale was sprinkled with 10 L of water (simulating

rainwater leaking into the damaged bale). Total PE content in low-quality haylage was examined and compared with high-quality haylage.

To isolate PEs from the matrix, direct extraction (to determine free PEs) and acid-based hydrolysis (to determine total PEs) were used. To determine the PEs, an optimized and validated method of ultra-performance liquid chromatography in combination with triple quadrupole mass spectrometry (ULPC-MS/MS) was used. Separation was done on an Acquity BEH C18 (50 x 2.1 mm; 1.7 μ m) analytical column.

Results and discussion

Differences were found in the PE profile and content among the individual tested clover materials. A statistically significant difference in PE content was found between the two species of clover (Figure 1).

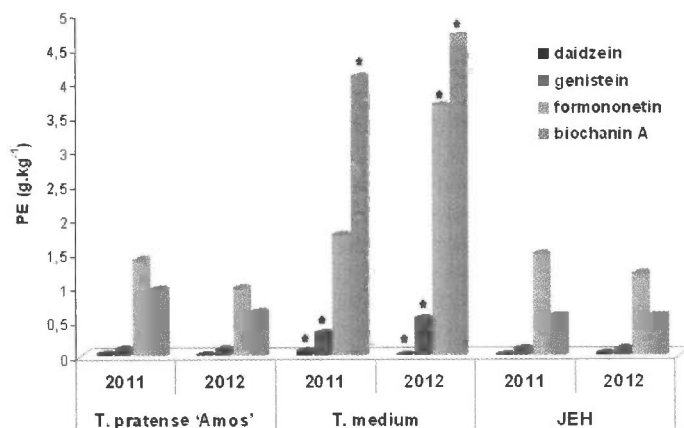


Figure 1. Comparison of phytoestrogen (PE) content in *Trifolium pratense*, *T. medium* and their hybrid (JEH) in the two-year experiment. * Statistically significant difference.

Plants in the hybrid (JEH) population contained a statistically inconclusive difference in PE content in comparison with *T. pratense* and a lower PE content than did *T. medium*. A higher content of biochanin A was observed in *T. medium* than in the hybrid JEH plants, and in the latter there was, by contrast, a higher content of formononetin.

Breeding material founded on *T. pratense* x *T. medium* hybrids is a rich source of genetic variability for *T. pratense*. That material's distinct DNA content had been analysed by flow cytometry and cytology (Řepková *et al.*, 2012) and was characterized by increased variability in morphological and agronomic traits (Jakešová *et al.*, 2011). The newly cultivated plants with the JEH designation were used to breed the new variety 'Pramedi'. The 'Amos' variety was used to stabilise the genomes of the hybrids by repeated open pollination with the JEH genotypes. The Czech Plant Variety Office granted rights for the new variety in 2013 (variety number TPM14855 and variety code 5082339).

A comparison of total PE content in high-quality (HQH) and low-quality (LQH) haylages (each material was always analysed from the upper layer, middle layer and at the centre of the bale) showed a slightly higher PE content in HQH samples from Závěšice in 2012. No significant difference was observed in PE content between HQH and LQH from Lukov in either harvest (2011 or 2012). A higher PE content was noticed in 2012 for both HQH and LQH samples in both studied areas (Figure 2).

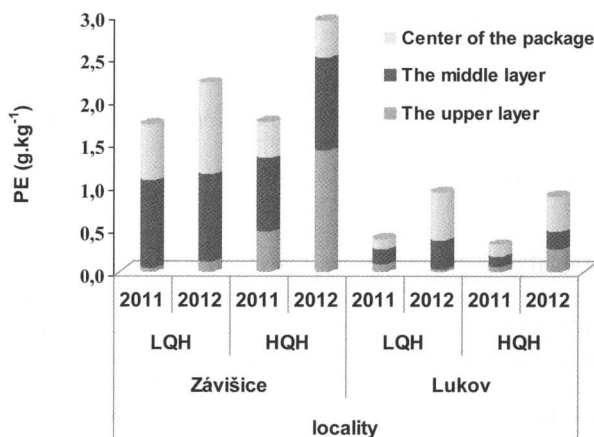


Figure 2. Comparison of total PE content in high-quality (HQH) and low-quality (LQH) haylages from Závišice and Lukov in the two-year experiment.

Comparison of PE content in individual layers of the bales demonstrates that the middle layer of the bale has the richest PE content in both HQH and LQH. Only in one instance was PE content higher in the upper layer of the bale, namely in the LQH sample from Závišice from 2012. The overall higher PE content in haylage from Závišice is primarily owing to the higher representation of red clover in the grass stand there (about 70%).

Conclusions

A statistically significant difference in phytoestrogen content was found between the individual species of clover. Plants in the hybrid JEH population contained a statistically inconclusive difference in phytoestrogen content in comparison with *T. pratense*. In high- and low-quality haylage samples, a significantly higher PE content was found in the sample from Závišice (2011 and 2012 harvests).

Acknowledgements

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