Review on Agriculture and Rural Development 2021 vol. 10 (1-2) ISSN 2677-0792
DOI: 10.14232/rard.2021.1-2.176-180

TOTAL SAPONIN CONTENT (TSC) OF DIFFERENT ALFALFA (*MEDICAGO SATIVA* L.) CULTIVARS CULTIVATED IN A FIELD EXPERIMENT

BALÁZS NAGY, PÉTER MAKLEIT

University of Debrecen, Faculty of Agricultural and Food Sciences and Environmental Management, Institute of Plant Sciences, Department of Applied Plant Biology

*Corresponding author: nagybalazs1205@gmail.com

ABSTRACT
Saponins are secondary metabolites produced by various plants. These compounds have important role in the defence system. The word saponin refers to a group of different chemical compounds. Basically, sugar conjugates of triterpenoids or steroids are called saponins. Triterpene-type saponins are more specific among dicotyledonous plants, while steroid-type saponins are more characteristic of plants belonging to the monocotyledonous taxonomic group. Alfalfa is a large-scale cultivated and foraged fodder plant in Hungary. In the defence mechanism of alfalfa, saponins also play an important role. However, large amount of saponins can be toxic in animal fodder, especially in the poultry farming and piggery. As a dicotyledonous plant, the alfalfa saponins are mainly triterpenoid type. In our study we measure the total triterpenoid saponin content and leaf stem ratio of field cultivated alfalfa cultivars. Samples were collected from a randomized block design experiment, planted in the Demonstration Garden and Arboretum of Institutes of Agricultural Research and Educational Farm, in Debrecen, in 2018. Three different cultivars were investigated, and the samples were collected three different times of the growing season at growing stage of early flowering, for three years (2018-2020.). There was no difference in the total saponin content (TSC) of examined varieties on the average of all measured data, although the sampling time showed significant effect on TSC. Our results attract attention to the fact that ageing of alfalfa stands can cause increase in TSC.

Keywords: Alfalfa, Saponin, Total Saponin Content, TSC

INTRODUCTION

Saponins are secondary metabolites produced by various plants and some inferior animal species. Primarily these compounds play important role in the defence system of plants or – in some cases– animals (e.g. sea cucumber). The term saponin refers to a group of natural compounds consisting of a non-polar aglycone of isoprenoidal origin (so-called genin or sapogenin) and one or more sugar units covalently linked thereto. In case of triterpenoid saponins, based on the number of sugar molecules attached to the aglycone backbone, we can distinguish between mono- and bidesmosidal saponins (AZIZ ET AL., 2019). The properties of their structural elements (polar and non-polar units) cause their soap-like behavior in aqueous solutions. The word saponin is derived from the Latin word "sapo", which also refers to the ability of compounds to form a stable foam when being shaken in aqueous solution (AUGUSTIN ET AL., 2011). Saponins have a variety of properties: they have sour or sweet taste, they also act as emulsifiers, they have a medicinal and haemolytic effect, and their antibacterial, insecticidal and molluscicidal properties are also known. Due to their properties, they are widely used in the production of beverages and confectionery, cosmetics and pharmacological products (VINCKEN ET AL., 2007). Because of their toxic effects on fish, Australian natives have favoured the use of saponin-containing plants in fishing (MILIGATE – ROBERTS, 1995). Higher saponin concentrations could be observed in plant tissues, which are favoured by various pests and pathogens (WINA ET AL., 2005). Some other factors also have impact on the saponin content of an individual plant. For example the temperature of the environment, the nutrient content of

Review on Agriculture and Rural Development 2021 vol. 10 (1-2) ISSN 2677-0792
DOI: 10.14232/rard.2021.1-2.176-180

the soil, the amount of water and light available or even the adjacent plants (SZAKIEL ET AL., 2011).

Alfalfa (*Medicago sativa*) is one of the largest fodder crops grown in Hungary, therefore it is essential to know the various content parameters of the plant. Saponins are one of the secondary metabolites of alfalfa. In alfalfa we can find triterpene type saponins. In the case of alfalfa, several sapogenins have already been isolated (e.g.: medicagenic acid, zanhic acid, hederagenin, etc.)(PECETTI ET AL., 2006). The most common monosaccharide side chains, which can attached to the alfalfa sapogenins, are galactose, glucose, and rhamnose or could be arabinose and xylose, but these last two are less important (CHEEKE, 1971). Saponins are considered to be an antinutritive factor in alfalfa. Early studies in the 1940's and 1950's suggested a link between alfalfa saponins and ruminant bloating. However, it is concluded that saponins do not contribute to pasture bloat in the case of ruminants (MAJAK ET AL., 1980). In addition, saponins may play a role in reducing methane production in ruminant species (KOZŁOWSKA ET AL., 2019). On the other hand, saponins could be undesirable components or even could be toxic for monogastric animals (PLEGER ET AL., 2019).

In our experiment, we wanted to find out which cultivar of the 3 observed (Tápiószelei, Hunor-40, Danubia) has the highest total saponin content (TSC) and whether the time of harvest has an impact on the TSC.

MATERIALS AND METHODS

Samples were collected at different times of each year (2018, 2019, 2020) from a randomized block design experiment, planted in the Demonstration Garden and Arboretum of Institutes of Agricultural Research and Educational Farm, in Debrecen, in 2018. In the first year there were 2 sampling dates, in the second and third years 3-3 sampling were performed. For the quantification of total saponin content we used the method developed by OLESZEK AND STOCHMAL (2002). As a first step, samples were dried at 65 °C then the samples were ground. In the solid phase extraction part 1 gram of each sample was used. 10 ml of 70% methanol was added to the samples and then the samples were shaken for one hour period. After that the samples were filtered using filter paper. Thereafter the filtrates were evaporated until 2-3 ml of aqueous phase remained in the evaporating flasks. The samples were then loaded onto C18 cartridges. After the sample diffused into the cartridge, the following was passed through it: 10 ml of distilled water, 5 ml of 40% methanol, 5 ml of concentrated methanol. The last methanol phase was collected in evaporating flasks and evaporated to dryness. The dry samples were collected with 1 ml of concentrated methanol.

The extraction procedure was followed by measurement with a photometer. The procedure we used was developed by LE ET AL. (2018). 0.025 ml was used from the previously collected samples. The samples were placed in a 65 °C water bath for 5 minutes to allow the methanol to evaporate. 0.5 ml of 4% ethanolic vanillin solution and 2.5 ml of 72% distilled aqueous sulfuric acid solution were added to the samples and then the samples were placed in a 60 °C water bath for 15 min. Finally, the samples were photometrized at the wavelength of 560 nm against a blank solution which solution contained 0.5 ml of 4% ethanolic vanillin solution and 2.5 ml of 72% distilled aqueous sulfuric acid solution. A calibration line using aescin was created previously and from the equation of the calibration line we deduced the total saponin content of the samples.

Statistical analyses were carried out by applying SPSS 23.0 version. One-way and two-way ANOVA were used, the groups were separated with Duncan test.

Review on Agriculture and Rural Development 2021 vol. 10 (1-2) ISSN 2677-0792
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RESULTS

There was no difference in the TSC of examined varieties on the average of all measured data (Hunor 40: 0,37±0,05 m m^{-1} %; Tápiószelei 1: 0.34±0.04 m m^{-1}%; Danubia: 0.39±0.06 m m^{-1}%). According to the two-way ANOVA, from the given factors: variety, sampling time and their interaction, only the sampling time showed significant effect on TSC (sig. Variety: 0.800; Sampling: 0.020; Variety x Sampling: 0.166).

Table 1. Effect of sampling time on alfalfa's TSC (m m^{-1} %; ±SE) on the average of all varieties' data; (n=12)

Sampling time	TSC
2018.08.29. - late summer	0.30±0.06^{ab}
2018.10.18. - autumn	0.30±0.04^{ab}
2019.06.13. - early summer	0.31±0.07^{ab}
2019.08.15. - late summer	0.15±0.05^{a}
2019.10.01. - autumn	0.32±0.06^{ab}
2020.05.14. - spring	0.39±0.09^{b}
2020.08.06. - late summer	0.44±0.09^{b}
2020.09.10. - autumn	0.69±0.09^{c}

*Letters in the table indicate different groups.

It can be concluded that the lowest TSC, on the average of the examined varieties was measured in case of the samples collected on the 15th of August, 2019, and the highest was measured in the samples collected on the 10th of September, 2020 (*Table 1*).
There was no correlation between the season of the year (spring, early summer, late summer, autumn) and the measured TSC (Pearson correlation coefficient: 0.084).

Table 2. Effect of sampling times on TSC (m m^{-1} %; ±SE) of different examined varieties (n=4)

Sampling time / TSC	Hunor 40	Tápiószelei 1	Danubia
2018.08.29. – late summer	0.20±0.08^{a}	0.44±0.12^{b}	0.25±0.08^{ab}
2018.10.18. - autumn	0.30±0.06^{a}	0.27±0.04^{ab}	0.33±0.09^{ab}
2019.06.13. – early summer	0.40±0.14^{ab}	0.34±0.14^{ab}	0.18±0.02^{a}
2019.08.15. – late summer	0.19±0.06^{a}	0.06±0.01^{a}	0.22±0.19^{a}
2019.10.01. - autumn	0.23±0.06^{a}	0.49±0.15^{b}	0.24±0.05^{ab}
2020.05.14. - spring	0.53±0.23^{ab}	0.49±0.08^{ab}	0.32±0.11^{ab}
2020.08.06. - late summer	0.40±0.06^{ab}	0.27±0.08^{ab}	0.64±0.23^{bc}
2020.09.10. - autumn	0.70±0.15^{b}	0.53±0.20^{b}	0.87±0.14^{c}

*Letters in the table indicate different groups.

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Examining the effect of sampling time on TSC of varieties separately, the next findings can be concluded (*Table 2*):

In case of Hunor 40 variety there were no differences amongst the measured TSC in the given years, but in the implied years differences were found thanks to the ageing of the stand.

Varieties Tápiószelei 1 and Danubia showed extreme low TSC on the late summer of 2019. The effect of ageing was no detectable in the measured data of Táiószelei 1 variety.

In case of Danubia variety the ageing of the plant stand also caused increase of TSC, as in case of variety Hunor 40.

Insofar that we compare the TSC of different experimental years on the average of all data of the examined varieties, it can be concluded that lower TS-contents were characteristic to the years 2018-2019. In 2020 higher TS-contents were measured (2018: 0.30 ± 0.03^{a} m m^{-1} %; 2019: 0.26 ± 0.04^{a} m m^{-1} %; 2020: 0.51 ± 0.06^{b} m m^{-1} %; ±SE) (n=24-32).

DISCUSSION

As water supply of the experimental plants was adequate from year to year, by applying irrigation, the background of the differences can be the ageing of the experimental plants and the temperature differences of examined years. It is possible that the older plants were under greater pressure due to pathogens and pests. Increased saponin synthesis may be associated with plant defence.

In the third year of the experiment, the highest total saponin levels were measured (2018: 0.30 ± 0.03^{a} m m^{-1} %; 2019: 0.26 ± 0.04^{a} m m^{-1} %; 2020: 0.51 ± 0.06^{b} m m^{-1} %). This finding may suggest that increasing saponin content is associated with plant aging.

The measured TS-contents correspond to other investigations. Earlier results presented higher TS-contents (0,8-2,0 m m^{-1} %), (PEDERSEN AND WANG, 1971; MAJAK ET AL., 1980). Newer publications, like KOZŁOWSKA AND CO-WORKERS (2020) presented 0.07 and 0.33 m m^{-1} %. TSC of ten alfalfa cultivars.

Although no correlation was found between the sampling season and the total saponin content of the samples based on statistical evaluation in our experiment, the literatures found that total saponin content is low in spring and autumn and peaking in mid-summer (HOWARTH, 1988; PECETTI ET AL., 2006).

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DOI: 10.14232/rard.2021.1-2.176-180

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