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COUMESTROL CONTENT IN ALFALFA BREEDING POPULATIONS

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Aim. Alfalfa is a rich source of phytoestrogens, among them coumestrol which shows strong estrogenic activity that can adversely affect the health of domestic animals. The aim of the study was to determine the variation in coumestrol content in leaves of alfalfa breeding populations, present in the breeding program of Agricultural Institute Osijek in Croatia. **Method.** Twelve alfalfa populations were selected based on their high forage yield and good persistence. Coumestrol was extracted using acidified methanol as an organic solvent from lyophilized and ground alfalfa leaves, while for detection and quantification was used. **Results.** Significant differences were observed between the studied populations with average coumestrol content of 435.67 mg/kg of dry matter (DM). The highest content of coumestrol was determined in breeding population Rs-21 (619.53 mg/kg of DM). **Conclusions.** Populations Rs-33 and Rs-20 had the lowest coumestrol content (82.18 and 86.58 mg/kg, respectively) and present a potential breeding source for creating new contemporary cultivars with decreased coumestrol content.

Key words: alfalfa, coumestrol, HPLC system, population, variation.

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INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the most significant perennial forage legume in the production of high quality and nutritionally valuable fodder for livestock feed, primarily ruminants (Vasileva, 2013; Tucak et al, 2018). Despite its exceptional value in livestock systems, alfalfa, like many other legume species and varieties, may contain phytoestrogens, the most interesting of which is coumestrol, which express strong estrogenic activity that can adversely affect the reproductive health of domestic animals (Hloucalova et al, 2016; Reed, 2016).

Coumestrol content in alfalfa is highly variable and can range from less than 1 mg/kg of DM (dry matter) to over 600 mg/kg of DM, whereby the currently available literature data on the quantification of the coumestrol content, and other phytoestrogens, vary greatly and is very often inconsistent (Seguin et al, 2004; Seguin and Zheng, 2006; Reed, 2016; Tucak et al, 2018). In EU and US countries, alfalfa is mostly grown as a single crop, very rarely in pasture systems mixed with grasses, and is used to feed livestock in the form of hay, haylage or silage. In some areas of the world (Australia, New Zealand, Argentina), alfalfa is an important forage legume used for direct sheep, beef and dairy cattle grazing (Stjepanović et al, 2009). According to many authors, the presence of coumestrol in alfalfa with levels above 25 mg/kg of DM is a potential barrier to its widespread use in grazing systems since it can lead to a decrease in the reproductive capacity of herbivores, primarily sheep, and therefore to a decrease in lambing rates if alfalfa is consumed immediately before and during the mating period (Smith et al, 1979; Cantero et al, 1996; Moravcova et al, 2002; Reed, 2016; Hloucalova et al, 2016; Cranston et al, 2017; Fields et al, 2018a). On the other hand, some studies cited that phytoestrogens in alfalfa showed only minimal effects on the fertility of sheep, and concluded that only pasture containing ~1000 mg/kg of DM will inhibit estrus and ovulation, and pasture containing ~200–400 mg/kg of

DM will only depress ovulation (Ruttle et al., 1968; Kelly et al, 1976; Scales et al, 1977; Sormunen-Cristian et al, 1998).

In *Medicago* species, coumestrol production is less influenced by genetic factors, and plant selection/ breeding usually will not be effective for controlling its content (Reed, 2016). Wide variation in coumestrol content is affected by numerous factors such as environmental conditions (temperature, rainfall, humidity, sunlight), cultivar, stressed conditions (especially by fungal diseases, insect infestation and nutrient deficiencies), stages of maturity, plant parts analyzed, management strategies and stand age of crops (Seguin et al, 2004; Aragadvay-Yungán et al, 2017; Fields et al, 2018b; Fields et al, 2019; Tucak et al, 2020). Fungal pathogens are considered as the main factor that increases coumestrol content in alfalfa. The more common foliar and stem diseases of alfalfa present in the field are common or Pseudopeziza leaf spot, Stemphylium leaf spot and spring black stem (Fields et al, 2018). Reduced coumestrol accumulation could be achieved by creating and selecting new modern yielding and fungal resistant alfalfa cultivars.

The aim of our research was to determine the variation in coumestrol content in field grown alfalfa breeding populations, created at the Department of Forage Crops Breeding and Genetics of the Agricultural Institute Osijek, Croatia, as initial screening in our breeding strategy in order to select potential materials with lower coumestrol content.

MATERIALS AND METHODS

Plant material and field experiment. Twelve breeding populations (Rs-5, Rs-9, Rs-17, Rs-20, Rs-21, Rs-33, Rs-47, Rs-50, Rs-MT, Rs-PS, L-14/1, L-16/18), from a total of 50 tested materials, were selected for the study based on their high forage yield and good persistence assessed over a two-year period (2014, 2015). All alfalfa material was obtained as part of the continuous long-term breeding program of perennial legumes at the Department of Forage Crops Breeding and Genetics of the Agricultural Institute Osijek, Croatia. The field experiment was conducted in the alfalfa breeding nursery, established on March 10 2014, and laid out under randomized complete block design (RCBD) in four replications. The basic nursery plot (4 rows, 1.6 × × 6 m) included 52 spaced plants with intra-row spacing of 50 cm and inter-row spacing of 40 cm (208 plants/population in total).

Sample collection and quantification of coumestrol in alfalfa populations. Leaf samples were collected from all 12 selected breeding populations from the established alfalfa nursery in July 2016. Leaf samples (100 g) were collected from 10 randomly selected plants of each population from two replications in the vegetative phase of the plants (late bud to early flower). The samples were frozen at −80 °C, lyophilized, ground to a powder from which coumestrol was extracted and quantified by Reverse phase (RP)-HPLC analysis, using a Perkin Elmer LC 200 HPLC system liquid chromatograph, with DAD detector and RP-C18 column, according to the methodology previously described in detail in Tucak et al (2020).

Statistical analysis. Statistical analyses were performed using STAR v. 2.0.1 software (IRRI, 2013). Data on coumestrol content were subjected to one-way analysis of variance (ANOVA). Fisher's protected least significant difference (LSD) was used to separate means when the ANOVA was significant at the 0.05 and 0.01 probability level.

Climate conditions. Data for mean monthly temperatures and total monthly precipitation for the alfalfa growing season (March – October) during the study period (2016) and a long-term average (LTA, 1971–2000) for the experimental location were obtained from Meteorological and Hydrological Service of the Republic of Croatia.

The average monthly air temperatures during the alfalfa growing season were higher for the majority of the months if compared to the long-term monthly average temperatures (Fig. 1, *a*). In 2016, the average air temperature was higher by 0.8 °C, compared to the long-term average temperatures.

A higher total amount of precipitation was recorded in all months of the studied year if compared to the long-term average, excluding April and September when the total amount of precipitation was slightly higher during the long-term average (Fig. 1, *b*). The particularly rainy month in 2016 was July, which had 40 % more precipitation indicating humid weather conditions, compared to the same month of the long-term average. More precipitation was recorded during the growing season of 2016 (561.9 mm), by 16.7 % more in comparison to the long-term average (467.8 mm), which indicates that weather conditions during the alfalfa growing season of 2016 were more humid and moderately warm.

RESULTS AND DISCUSSIONS

Analysis of variance (ANOVA) of the obtained values for coumestrol content in DM showed statistically significant differences (at the probability level

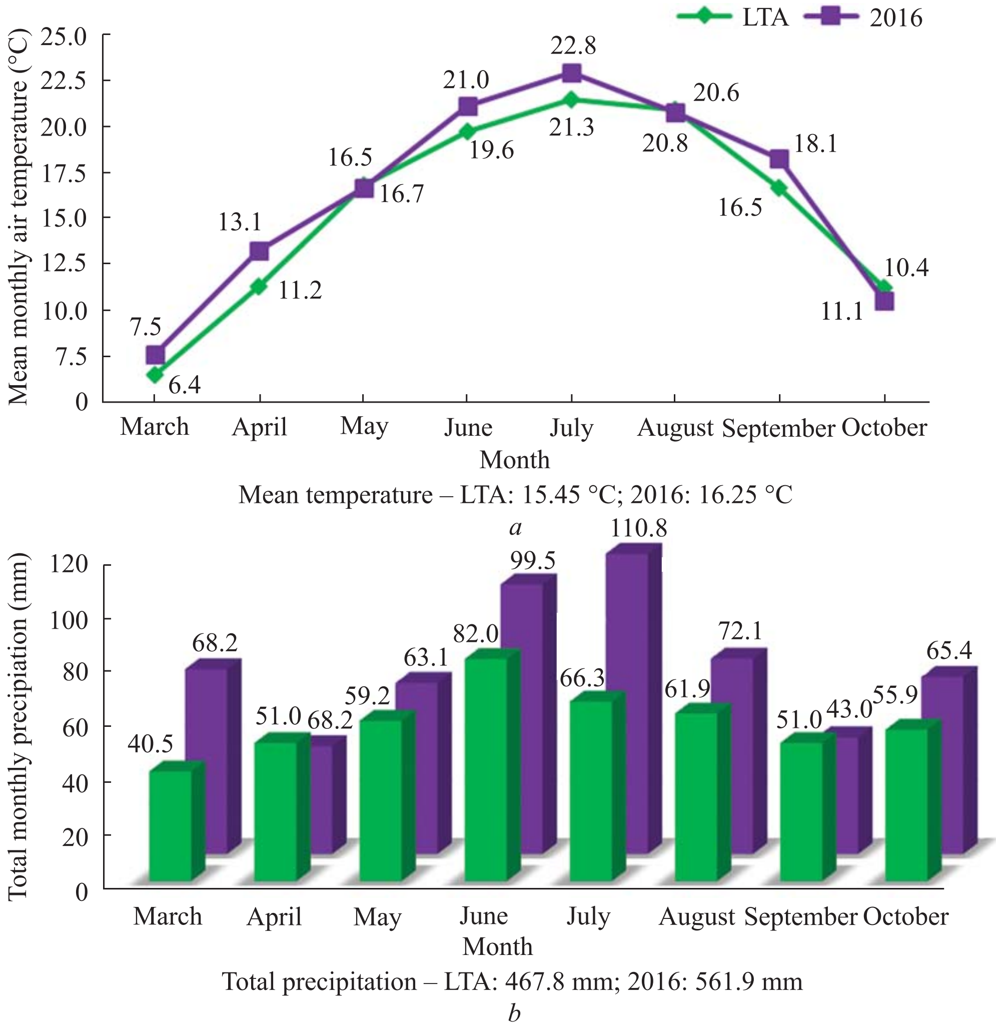

Fig. 1. Mean monthly air temperatures (*a*) and total monthly precipitation (*b*) for the alfalfa growing season (March – October) during the study period (2016) and a long-term average (LTA, 1971–2000) for the experimental location (Source: Meteorological and Hydrological Service of the Republic of Croatia)

$p = 0.01$) between the studied alfalfa breeding populations (Table). The average coumestrol content of all observed populations was 435.67 mg/kg of DM, and the coefficient of variation (CV) was 2.38 % (Fig. 2). The lowest coumestrol content was obtained from the breeding population Rs-33 (82.18 mg/kg of DM), which was not significantly lower than the value determined for the Rs-20 population (86.58 mg/kg of DM). The coumestrol content found in these populations was 5 times lower than the average content for all populations. Contrary, breeding population Rs-21 had the highest coumestrol content (619.53 mg/kg of DM).

Earlier research also showed that alfalfa cultivars may vary widely in coumestrol content and that the variation can be caused by plant genotype, stage of

Analysis of variance (ANOVA) for coumestrol content

Sources of variation	Sum of square (SS)	Degree of freedom (DF)	Mean square (MS)	F-value	ProbF
Repetition	1195.41	1	1195.41	11.04	
Population	807216.92	11	73383.36	677.91 **	0.001
Residual	1190.75	11	108.25		
Total	809603.08	23	35200.13		

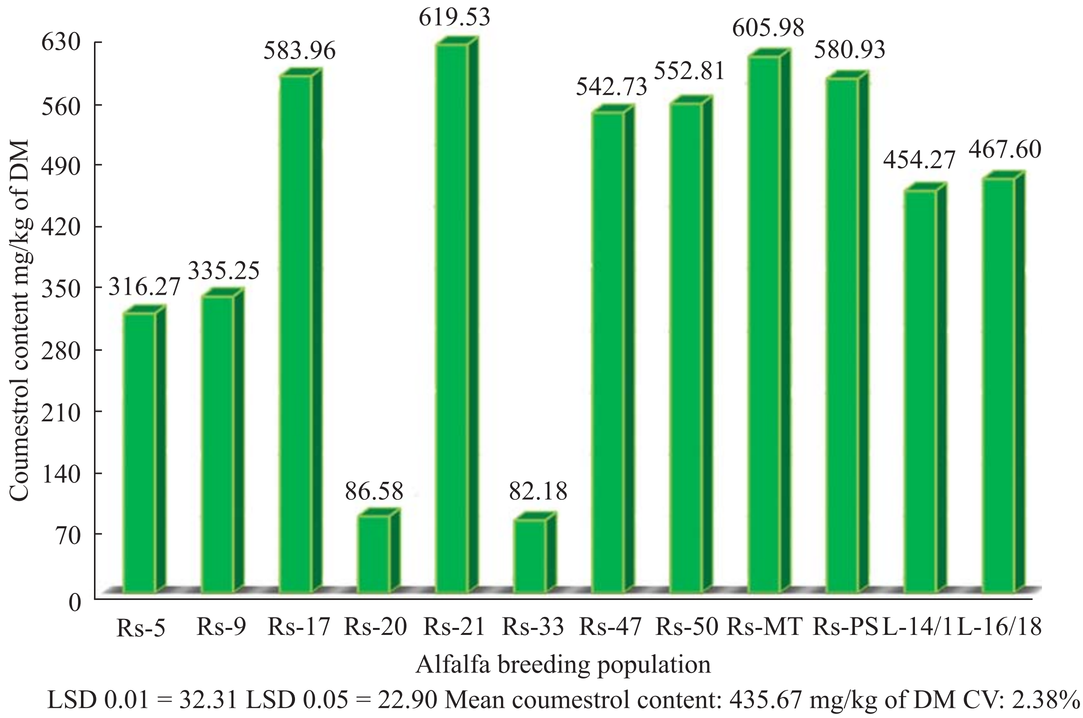

Fig. 2. Mean coumestrol content (mg/kg of dry matter – DM) in the leaves of alfalfa breeding populations

growth and/or environmental impact. Of the latter biotic stress is the most significant and commonly studied factor. Moravcova et al. (2004) evaluated the influence of several variables on the coumestrol content of alfalfa silages and determined that the content varied with cultivars and maturity of plant. The authors reported average coumestrol concentrations of 100 mg/kg of DM, with a 10 % variation between two cultivars.

Seguin and Zheng (2006) determined the concentrations of three phytoestrogens in the seeding and post-seeding year at two sites and observed the large variation for coumestrol content between both harvests and sites, despite the lack of differences between ten field-grown alfalfa cultivars. These authors reported an average coumestrol content of 141 mg/kg of DM. Seguin et al. (2004) studied the effect of plant maturity and herbage components on contents of different alfalfa phytoestrogens and reported that coumestrol contents ranged between 50 and 135 mg/kg of DM, were high at late vegetative and late seed pod stage, and low at early flower stage. Significant differences were observed between canopy segments with coumestrol contents being greatest in the top segment, however no differences were observed between plant parts.

Fields et al (2018b) assessed individual effects of agronomic factors (cutting frequency, development stage, and cultivar) in both the field and greenhouse grown alfalfa on coumestrol content and concluded that fungal pathogens infection and inoculation treatment significantly increased coumestrol content up to 396 mg/kg of DM, while the development stage of the plant had no effect. Elevated levels of coumestrol in alfalfa herbage were also observed because of pea aphid herbivory.

A substantial higher average coumestrol content was found in our study (435.67 mg/kg of DM), except for the populations Rs-33 and Rs-20 (82.18 and 86.58 mg/kg of DM, respectively), in comparison with the results of the abovementioned authors. One of the possible causes for this could have been the weather conditions during the conducted research. Good weather conditions during the 2016 alfalfa growing season, favorable air temperatures and higher total amount of precipitation, compared to the long-term average (Fig. 1, *a*, *b*), could have contributed to the increased coumestrol accumulation in the studied populations. Fields et al (2019) obtained similar results while developing a model to predict coumestrol content in non-irrigated alfalfa crops using weather variables and concluded that rainfall is a major component in the model. According to these authors if total rainfall was between 61 and 131 mm during alfalfa regrowth period coumestrol content is likely to be moderately high (58.4 mg/kg of DM), while with total rainfall over 131 mm and more than

17 days with relative humidity above 95 % coumestrol level was predicted to be high (163 mg/kg of DM). Likewise, high rainfall and humidity conditions during July, when leaf samples were collected in this experiment, could have caused fungal foliar diseases. These diseases according to Fields et al (2019) can cause a defense reaction leading to elevated coumestrol levels in alfalfa. Sampling of leaves at a later vegetative stage may have also contributed to the increased accumulation of coumestrol in our study, which is consistent with previously reported results (Hanson et al., 1965; Seguin et al., 2004).

Fields et al (2018b) reported differences in coumestrol content in ten alfalfa cultivars with levels in all cultivars posing a risk for ewe reproductive performance, and recommended that alfalfa breeding should be focused on fungal resistance. *Medicago* spp. genotypes with low coumestrol content tested for fungal resistance according to Barbetti (2006) should be included as parental material in annual *Medicago* spp. breeding programs. Populations tested in our experiment with low coumestrol content, which already showed high forage yield and good persistence, will be further included in breeding program for creating cultivars with decreased phytoestrogen content.

CONCLUSIONS

A significant variation in coumestrol content was found between the investigated field grown alfalfa breeding populations (82.18 to 619.53 mg/kg). The two populations Rs-33 and Rs-20 showed significantly lower coumestrol content (82.18 and 86.58 mg/kg of DM, respectively) than the other observed alfalfa materials and they present potential breeding source for creating new cultivars with decreased coumestrol content.

Adherence to ethical principles. This article does not relate to any studies using humans and animals as investigation subjects.

Conflict of interest. The authors deny any conflict of interests.

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Вміст куместролу в селекційних популяціях люцерни

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Мета. Люцерна – це багате джерело фітоестрогенів, серед яких куместрол має найвищу естрогенну активність, що може мати негативний вплив на здоров'я свійських тварин. **Мета** дослідження полягала у визначенні різниці між показниками вмісту куместролу в листі люцерни з селекційних популяцій, включених до селекційної програми Інституту сільського господарства в м. Осієк, Хорватія. **Метод.** Дванадцять популяцій люцерни було обрано на основі високої урожайності цієї кормової культури та хороших показників стійкості. Задля виявлення і кількісного визначення куместрол виділяли з ліофілізованого та подрібненого листя люцерни за допомогою підкисленого метанолу в якості органічного розчинника. **Результати.** Було встановлено суттєву різницю між досліджуваними популяціями, середній вміст куместролу становив 435,67 мг/кг сухої речовини (СР). Найвищий вміст куместролу було встановлено у селекційній популяції Rs-21 (619,53 мг/кг СР). **Висновок**. Популяції Rs-33 і Rs-20 мали найнижчий вміст куместролу (82,18 і 86,58 мг/кг, відповідно), вони є потенційним джерелом селекції для створення сучасних сортів зі зниженим вмістом куместролу.

Ключові слова: люцерна, куместрол, система HPLC, популяція, варіаційні зміни.

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