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FOLIAR APPLICATION OF SELENIUM, ZINC AND COPPER IN ALFALFA *(Medicago sativa L.)* BIOFORTIFICATION

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ABSTRACT

Biofortification of forage crops has an important role in improving the quality of plants used for animal nutrition. The field experiments were conducted in three consecutive years in Subotica, Serbia, in order to investigate the effect of Se, Zn, and Cu foliar fertilization on the yield, Se and Zn contents and nutrient efficiency, as well as on other mineral compositions of alfalfa hay. The treatments were as follows: i) control without fertilization, ii) 5 g Se ha^{-1}, iii) 10 g Se ha^{-1}, iv) 0.5 kg Zn ha^{-1}, v) 1 kg Zn ha^{-1}, vi) the combination of these two elements (0.5 kg Zn ha^{-1} and Se 10 g ha^{-1}) and vii) 2% Cu solution. The application of Se, Zn, and Cu had no effect on dry yield or on crude protein, P, K, Ca, Mg, Fe, Mn, Mo, and Co contents in alfalfa hay. However, Se, Zn, and Cu fertilization significantly increased the contents of Se, Zn, and Cu in alfalfa hay. The results showed that Se and Zn contents in plant biomass were significantly correlated with the applied doses of Se (r=0.99) and Zn (r=0.99). The production years of alfalfa and the weather conditions proved to be significant factors in fertilization efficiency.

Keywords: Animal feed, fertilization, fertilization efficiency, microelements.

INTRODUCTION

Selenium (Se) has not been identified as an essential element for plants (Terry et al., 2000), but it is very important for animal's health. The adequate level of Se (0.1 mg Se kg^{-1} DM) in the animal diet is necessary (Gupta, 2008); otherwise, the deficiency may cause a several of disorders such as poor growth, white muscle disease, or infertility (Fisher, 2008). Selenium has a high antioxidant ability and it is integrated into glutathione peroxidase (GPHPx), an enzyme responsible for maintenance of red blood cells and cell membranes (Gupta and Gupta, 2002). According to Gupta and Gupta (2002), the soils containing more than 0.6 mg Se kg^{-1} dry matter (DM) will produce forage crops which can satisfy the nutritional needs of animals. In previous studies in the Balkan region, it has been indicated that the soils are deficient in Se (Manojlović and Lončarić, 2017). In the soil samples collected from different forage crops in North and East Serbia, the Se contents were below the specified level, in the range of 0.14-0.57 mg kg^{-1} (Marijanušić et al., 2017). Also, an inadequate level of Se was measured in the blood of sheep and cows from the Western Balkan region, as reported by Antunović et al. (2010) and Ademi et al. (2015).

Zinc (Zn) represents a vital element in the nutrition of plants, animals, and humans. Zn deficiency in animals may result in anorexia, abnormalities of the skin, skeletal or reproductive disorders (Suttle, 2010). The Zn requirements for ruminants and monogastric animals are different and range from 40-50; 40; 50-100, and 29-44 mg kg^{-1} DM for cows, sheep, pigs, and chicks, respectively (Fisher, 2008). The symptoms of Zn deficiency usually occur due to an insufficient level in feed. The northern part of Serbia (Vojvodina Province) is considered to be a Zn deficient area (Manojlović and Singh, 2012). In a study conducted in Serbia, the Zn contents in most of the soil samples (total n=157) were above the specified limit of 0.5 mg kg^{-1} (DTPA extractable Zn), and only in 13% of the samples mostly collected in the Vojvodina Province below this limit (Nikolic et al., 2016). According to Marijanušić et al. (2017) the measured Zn contents in all of the samples of forage crops (alfalfa and grasses) in Vojvodina and East Serbia were below 15 mg kg^{-1}.

Copper is an integral part of a large number of enzymes (ferroxidase, lysyl oxidases, tyrosinase, etc.) necessary for the normal functioning of the organism. Copper deficiency in livestock nutrition may lead to anemia, osteoporosis, depigmentation, or other disorders (Suttle, 2010). The sufficiency levels of Cu in crops used for livestock nutrition are 4-10 mg kg^{-1} for cattle; 6-10 mg kg^{-1} for sheep, or an even higher level of 60 mg kg^{-1} for post-weaning pigs. Cu content in fodder plants in Serbia varies depending on the site and the plant species and is generally below the established limits for livestock production (Manojlović and Singh, 2012; Marijanušić et al., 2017).

Alfalfa is one of the most valuable forage crops used for animal feed. The importance of alfalfa is due to its high and stable yields of nutritious feed. In addition to a high content of crude proteins, contains high levels of microelements that are important for the growth and development of animals (Radović et al., 2009).

Forage crops represent the natural source of minerals in livestock nutrition (Suttle, 2010). The natural deficiency could be compensated for through numerous measures: biofortification of forage crops (Novoselec et al., 2018), or treatment of animals with injections, mixing with food, etc. (Fisher, 2008). However, a great number of studies have demonstrated the advantage of the organic source of microelements over the inorganic. The organic sources of Se were more efficient in increasing blood Se and blood GPHPx of lambs (Qin et al., 2007), boars (Petrujkić et al., 2014), and beef cows (Slavik et al., 2008). Ao et al. (2009) reported that the antagonism of Zn and Cu in the diet of chicks could be avoided by using the organic sources of these elements. Hall et al. (2013) found out that Se fertilized alfalfa (22.5; 45.0; 89.9 g Se ha^{-1}) could improve Se status in the blood of weaned beef calves. Numerous studies have reported that foliar fertilization is an important tool in biofortification of forage crops with Se and Zn (Ceylan et al., 2009; Nawaz et al., 2016; Wang et al., 2013). On the other hand, the literature shows limited information related to the foliar Cu fertilization of forage crops.

Considering the above mentioned facts that Se, Zn, and Cu represent the vital microelements for livestock health maintenance; that forage crops as organic sources of minerals are deficient in Se, Zn and Cu on the territory of Serbia; and that foliar fertilization is an important measure to improve the status of these elements in forage crops; this study was conducted to investigate 1) the effect of foliar fertilization with Se, Zn, and Cu on alfalfa yield and the contents of Se, Zn and Cu in alfalfa biomass; 2) the effect of foliar fertilization on the content of other macro and micro elements; and 3) the efficiency of applied fertilizers.

MATERIALS AND METHODS

Experimental site

The studies were conducted at a dairy farm in the vicinity of Subotica, Serbia (46°3'39.92"N, 19°32'3.77"E) under rainfed conditions from 2014 to 2016. Subotica is situated in the north part of Serbia and the south part of the Pannonian Basin, near the Serbian-Hungarian border. The soil of the experimental site is Calcic Chernozem and the basic physical and chemical properties of the soil are given in Table 1. The high content of $CaCO_3$ is followed by a high pH of the soil. The studied soil is in the class of medium humous, optimally provided with phosphorus i.e. moderate provided with plant-available potassium. The concentrations of total (Se and Mo) and plant-available macro- (Ca and Mg) and microelements are shown in Table 2. Given the high pH value of the soil, the lack of certain microelements was expected, however, the experimental plot is sufficiently provided with available Zn and Cu (Lindsay and Norvell, 1978). The determined selenium content (0.26 mg kg^{-1}) in soil is below the suggested level (0.6 mg kg^{-1}) for the production of forage crops (Gupta and Gupta, 2002).

Table 1. Basic chemical and physical properties of soil before setting up the experiment

	Chemical	**properties**					
cm depth	**pH in KCl**	**pH in H_2O**	**% $CaCO_3$**	**% Organic matter**	**% Total N**	**mg 100g^{-1} AL-P_2O_5**	**mg 100g^{-1} AL-K_2O**
0-30	7.39	8.02	19.3	2.53	0.13	21.2	14.0
	Physical	**properties**					
cm depth	**% coarse sand**	**% fine sand**	**% silt**	**% clay**			
0-30	3.8	44	32.6	19.6			

Table 2. Total and plant-available contents of macro- and microelements in soil

	Plant	**available**						**Total**	
depth	**% Ca**	**% Mg**	**mg kg^{-1} Fe**	**mg kg^{-1} Zn**	**mg kg^{-1} Mn**	**mg kg^{-1} Cu**	**mg kg^{-1} Co**	**mg kg^{-1} Se**	**mg kg^{-1} Mo**
0-30	0.15	0.008	43.0	3.36	26.6	3.33	0.50	0.26	0.32

The total monthly precipitation and mean monthly temperatures during the experiment are given in Figures 1 and 2. Long term average (LTA) represents collected data about monthly temperatures and precipitations in the period 1981-2010 on the weather station Palić. The year 2014 (Figure 1) characterized by a higher rainfall compared to the LTA. However, the problem is the distribution of precipitation during vegetation, which is very unfavourable for spring sowing of alfalfa. The precipitation rate in March was almost twice lower (by

16.6mm) compared to the LTA. The second year of the experiment (2015) was distinguished by the average monthly temperatures at the level of LTA, with slightly higher temperatures in the period from June to September. Regarding the precipitation during the vegetation period, except for May and August, in most other months, it was significantly lower than the average.

In the third year, the temperature conditions were at the same level or slightly higher than the LTA. During the period from May to June, the precipitation rate was close to the LTA, i.e. significantly above from August to October.

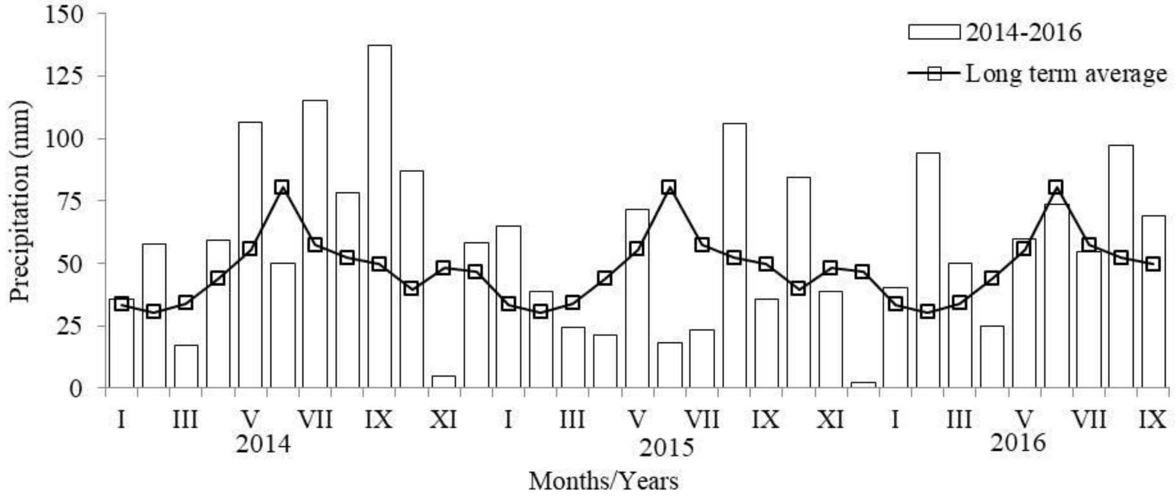

Figure 1. Monthly precipitations in Subotica

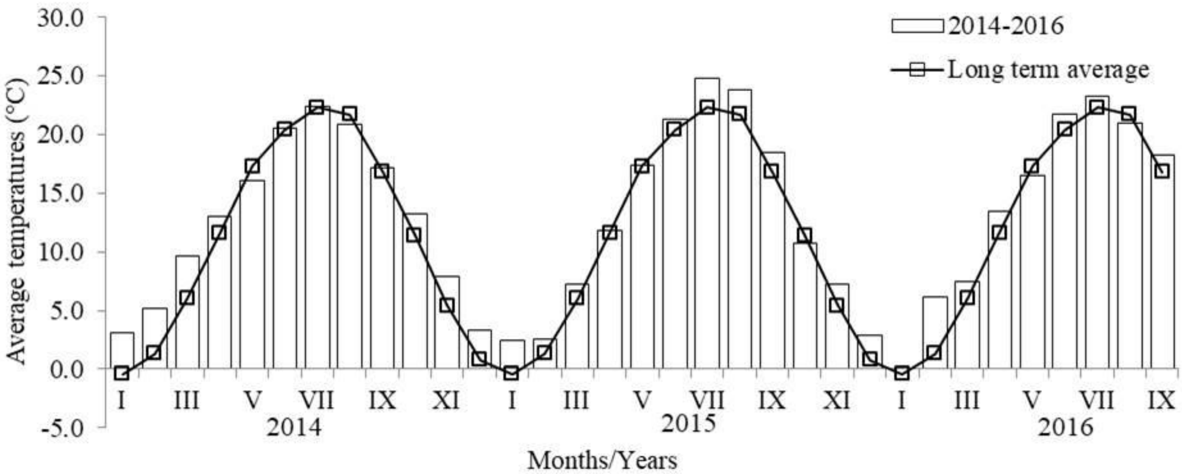

Figure 2. Mean monthly temperatures in Subotica

Experimental design and treatments

The experiment was conducted as a randomized block design with four replicates and each individual plot was 5x1 m. The treatments were as follows: *i*) control without foliar fertilization, *ii*) Se at two rates of 5 and 10 g Se ha^{-1} (as sodium selenate, Na_2SeO_4), *iii*) Zn at the rates of 0.5 and 1 kg Zn ha^{-1} (as zinc sulphate heptahydrate, $ZnSO_4.7H_2O$), *iv*) 2% Cu solution (as cupric sulphate, $CuSO_4.5H_2O$) and *v*) the combination of Se and Zn (0.5 kg Zn $ha^{-1}$and Se 10 g ha^{-1}). The fertilization doses were divided into two foliar applications. The first application was carried out at a plant height of 10 cm, and the second application was seven days after the first. In order to avoid leaf burn, fertilization was performed in the late afternoon. Besides the fertilizer, an adhesive was added during the application. During the foliar fertilization, the plants on the control treatment were treated with distilled water and adhesive. The trial started at the end of March 2014. Sowing was performed by machine at a row spacing of 14.5 cm. The alfalfa cultivar was NS Banat ZMS II with the seeding rate of 20 kg ha^{-1}. At all experimental plots 50 kg N (as ammonium nitrate, 33% N), 70 kg P2O5, and 105 kg K2O ha-1 (as PK 20:30) was incorporated into soil at the time of sowing.

Sampling

A soil sample was taken before setting up the experiment (March 2014), from 0 to 30 cm soil depth.

Alfalfa was harvested at the early flowering stage; at a cutting height of approximately 8 cm. Alfalfa fresh yields were measured in 2014, 2015, and 2016, by harvesting 1 m^2 of each plot and weighing the samples in the field. The samples were dried to constant weight in an air dryer (70°C) and weighed again to estimate the percentage of dry matter. The dry matter yield was determined on the basis of fresh biomass yield and the percentage of dry matter.

Chemical analysis

The soil reaction was determined using a soil: water and a soil: 1M KCl ratio of 1:2.5 (Mettler Toledo, Five Easy FE 20); the content of $CaCO_3$ volumetrically (ISO 10693:2005) using a Scheibler calcimeter (Hedas, Serbia); the organic matter content was determined by the dichromate method (oxidation with 0.4N $K_2Cr_2O_7$, ISO 14235:2005). The available phosphorus (P) and potassium (K) in the soil samples were extracted with AL solution (0.1 M ammonium lactate and 0.4 M acetic acid, pH 3.75) and a soil: solution ratio of 1:20 (AL-method, Egner and Riehm, 1960). The phosphorus content in the extract was determined using a spectrophotometer (Shimadzu UV 2600, Japan) and the K content using a flame photometer (Jenway 6105, USA). The available contents of calcium (Ca), magnesium (Mg), Cu, manganese (Mn), Zn, iron (Fe), and cobalt (Co) were extracted using a buffered solution of diethylenetriaminepentaacetic acid - triethanolamine (0.005 M DTPA + 0.01 M $CaCl_2$ + 0.1 M TEA) and a soil: solution ratio of 1:2 (ISO 14870:2001). Measuring was conducted by an atomic absorption spectrophotometer (Shimadzu 6300, Japan). The reference material used for analyses was ISE 973 (Wepal, The Netherlands). Microwave digestion of soil (0.2 g) with hydrofluoric acid (1 ml) and nitric acid (2 ml) in a high-pressure system (UltraCLAVE Milestone, Italy) was performed to determine the total contents of Se and Mo. The contents were analyzed by ICP-MS (Agilent 8800 QQQ, USA). Two reference materials were used: DC73324 and NCS ZC73007 (National Analysis Center for Iron and Steel, China).

Since the foliar fertilization was applied only before the first cut, accordingly, the chemical analyses were done only in the samples from that cut. The samples from the first cut for two years (2014 and 2015) were analyzed for crude protein content (CP) and macro- and microelement concentrations (P, K, Ca, Mg, S, Se, Cu, Zn, Fe, Mn, Co, and Mo). A cutting mill (Retsch SM 100, Germany) with a heavy-metal free grinding tool was used to grind plant material into powder. Crude protein content was calculated as nitrogen content x 6.25. Nitrogen and sulfur (S) contents were measured using a CHNS analyzer (Vario MACRO Cube, Elementar Analysensysteme GmbH, Germany). In order to determine P and K, the plant samples were burned in an oven at 550°C and then digested with 25% HCl (high-temperature dry ashing, Kalra, 1998). The phosphorus content was measured by spectrophotometry while K content using a flame photometry method. Wet digestion with a mixture of nitric and perchloric acid (HNO_3, $HClO_4$ and plant in a 20:5:1 ratio) was used to determine the microelement contents in alfalfa samples (nitric-perhloric acid wet digestion in an open vessel, Kalra, 1998). Reference material was IPE 885 (Wepal, Netherlands) and the measuring was done by AAS. For the analyses of Se and Mo, the plant samples (0.2 g) were digested with 5 ml of nitric acid and 5 ml of ultrapure water in a high-pressure microwave digestion system (UltraCLAVE Milestone, Italy). The reference material used was wheat flour 1567a and apple leaves 1515 (National Institute of Standards and Technology, USA). The content of Se and Mo was determinated using the ICP-MS method.

Apparent recovery efficiency (ARE, %) was calculated separately for Se and Zn according to the following formula (Fixen et al., 2015):

$$\frac{\text{Element accumulation on fertilized treatments } (\text{g ha}^{-1}) - \text{element accumulation on control treatment } (\text{g ha}^{-1})}{\text{Amount of applied element } (\text{g ha}^{-1})} * 100$$

The results were subjected to a two-way analysis of variance ANOVA (treatments and years) whereas Fisher's test ($p < 0.05$) was used to detect the significant differences between the treatment means. The statistical analysis was performed using the STATISTICA 13.2 software.

RESULTS

Alfalfa yield

In the first year of the experiment (year of crop establishment) alfalfa achieved only two cuts. In subsequent years, annual dry yields were higher, due to more achieved cuts. Foliar application with Se, Cu, and Zn showed no significant effect on alfalfa dry yield compared to control in all years of the experiment (Table 3). The average dry matter yield in the first cut and the annual yield in 2016 were statistically higher than those achieved in 2014 and 2015. Also, statistically significant differences between the measured yields in 2014 and 2015 were observed.

Selenium and zinc content in alfalfa

Selenium fertilization increased the Se content and its accumulation in alfalfa biomass (Table 4). In comparison with control, a significantly higher Se content was measured on the 10 g Se ha^{-1} treatment in both years. The application of 5 g Se ha^{-1} increased the Se content by 70 and 56% in 2014 and 2015, but not significantly compared to control. Se accumulation was increased by 42 and 69% after the application of 5 and 10 g Se ha^{-1} in 2014, but not significantly compared to control. Significantly higher nutrient accumulation was measured in 2015 on the treatment with 10 g Se ha^{-1} by 149% compared to the treatment without fertilization. Correlation analysis showed that 1 g of Se applied per hectare increased the Se content by 0.40 mg kg^{-1} DM of alfalfa biomass in both years (Fig. 3). On all Se applied treatments, apparent recovery efficiency was higher in the second year compared to the first year of the experiment.

Table 3. Effect of fertilization with selenium, copper, and zinc on dry matter yield (t ha^{-1})

	Treatment	**Yield by cuts**				**Annual**
		I	**II**	**III**	**IV**	**yield**
2014	control	1.97	1.97	-	-	3.94
	Se 5g	1.62	1.71	-	-	3.33
	Se 10g	1.76	1.67	-	-	3.43
	Zn 0.5 kg	2.70	1.82	-	-	4.52
	Zn 1kg	2.25	2.16	-	-	4.41
	Cu 2%	1.51	1.94	-	-	3.45
	Zn + Se	2.21	1.87	-	-	4.08
2015	control	4.30	2.73	2.31	-	9.34
	Se 5g	4.80	2.90	1.90	-	9.60
	Se 10g	4.56	2.50	1.88	-	8.94
	Zn 0.5 kg	4.61	2.87	2.41	-	9.89
	Zn 1kg	4.76	2.96	1.88	-	9.60
	Cu 2%	4.10	2.65	1.93	-	8.68
	Zn + Se	3.99	2.97	2.34	-	9.30
2016	control	4.69	2.97	1.56	1.10	10.32
	Se 5g	5.21	3.57	2.14	1.60	12.52
	Se 10g	5.95	3.30	1.42	1.03	11.70
	Zn 0.5 kg	5.91	3.17	1.83	1.02	11.93
	Zn 1kg	5.04	2.77	1.91	1.05	10.77
	Cu 2%	4.40	3.09	1.79	1.51	10.79
	Zn + Se	4.71	2.68	1.65	1.35	10.39
	Annual	average				
	2014	2.00 C*	1.88B			3.88 C
	2015	4.45 B	2.80A	2.09 A		9.34 B
	2016	5.13 A	3.08A	1.76 A	1.24	11.20 A

*capital letters represent differences between years

Table 4. Effect of fertilization with selenium, copper, and zinc on Se and Zn content in alfalfa biomass (first cut)

	Treatments	**Se content**	**Se accumulation**	**ARE**	**Zn content**	**Zn accumulation**	**ARE**
		mg kg^{-1}	**g Se ha^{-1}**	**%**	**mg kg^{-1}**	**g Zn ha^{-1}**	**%**
2014	control	0.37 bc*	0.77	-	15.58 c	31.20 cd	-
	Se 5g	0.63 ac	1.09	6.4	17.54 c	28.39 d	-
	Se 10g	0.77 a	1.30	5.3	16.01 c	28.29 d	-
	Zn 0.5 kg	0.49 ab	1.46	-	33.11 b	91.70 ab	12.1
	Zn 1kg	0.38 bc	0.90	-	49.90 a	112.95 a	8.2
	Cu 2%	0.21 bd	0.33	-	18.05 c	27.16 d	-
	Zn + Se	0.53 ac	0.85	0.8	29.13 b	64.08 bc	6.6
2015	control	0.27 c	1.29 b	-	16.13 bc	68.90 b	-
	Se 5g	0.42 bc	1.93 b	12.8	15.18 bc	73.10 b	-
	Se 10g	0.68 ab	3.21 a	19.2	16.48 bc	75.09 b	-
	Zn 0.5 kg	0.28 c	1.44 b	-	20.08 ab	94.16 ab	5.1
	Zn 1kg	0.25 c	1.31 b	-	23.93 a	115.80 a	4.7
	Cu 2%	0.32 c	1.27 b	-	16.06 bc	64.91 b	-
	Zn + Se	0.75 a	3.48 a	21.9	19.88 ab	80.72 b	2.4
	Annual	average					
	2014	0.48 A**	0.94 B	4.17	25.62 A	54.82 A	8.97
	2015	0.43 A	1.99 A	17.97	18.25 B	81.81 B	4.07

*small letters represent differences between treatments within one year
**capital letters represent differences between years

In 2014, on the treatments with 0.5 and 1 kg Zn ha^{-1}, a significantly higher Zn content (by 113 and 220%) was measured compared to control. In the next year of the experiment, a significantly higher Zn content (by 48%) was measured only on the treatment with the higher Zn dose. When comparing Zn treatments (0.5 and 1 kg ha^{-1}) with control, higher nutrient accumulation (by 194 and 262%, respectively) was achieved in 2014 than in 2015 (by 37 and 68%, respectively). The effect of foliar applied Zn on its content in alfalfa biomass was different in two years. By applying 0.1 kg Zn ha^{-1}, the Zn content was increased by 34.32 mg Zn kg^{-1} DM in 2014 and by 7.80 mg Zn kg^{-1} DM in 2015 (Fig. 3). Apparent recovery efficiency was the highest in the first year of the experiment on the treatment with the lower Zn dose. On average, Zn fertilization was more efficient in 2014.

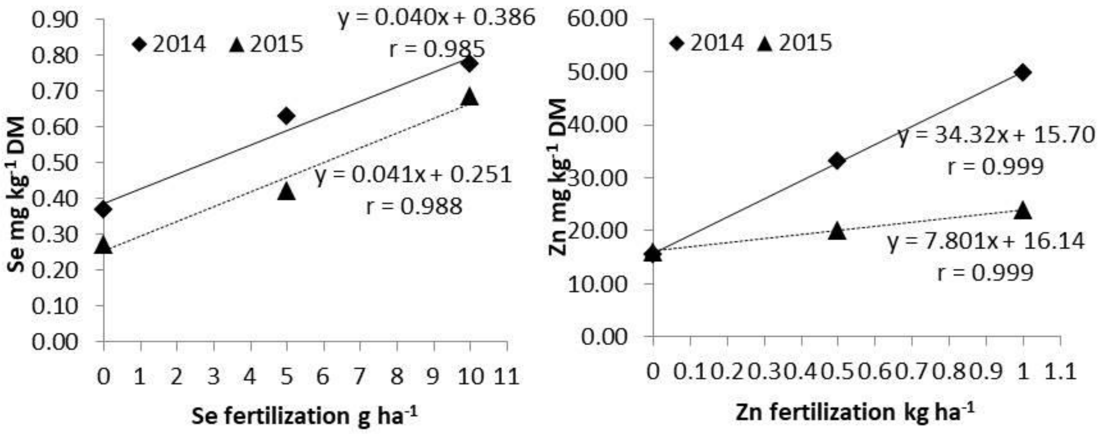

Figure 3. Correlation between Se fertilization and its content in alfalfa biomass and Zn fertilization and its content in alfalfa biomass

Fertilization with the combination of Se and Zn (Table 4) increased the Se content in both years (51 and 178%) compared to control, but it was significantly higher only in 2015. Also, the treatment significantly affected Se removal in 2015. As expected, this treatment positively affected the content of Zn in alfalfa biomass, but it was significantly higher only in 2014 (by 87%). On the other hand, there was no significant effect of Zn removal in both years. The combined fertilization with 10 g Se ha^{-1} and 0.5 kg Zn ha^{-1} did not significantly affect the content and nutrient accumulation of these elements compared to the treatments fertilized with 10 g Se ha^{-1} and 0.5 kg Zn ha^{-1}. Apparent recovery efficiency for Se was the highest in 2015 on the treatment with Se and Zn combined.

Macromineral contents in alfalfa biomass

Selenium and Zn fertilization showed no significant effect on the CP content in 2014 (Table 5). In the second year of the experiment, a significant difference was found between the control and Se+Zn treatment. In both years, the S content on the treatments with applied Se and Zn decreased compared to control, but not significantly. The exceptions were the treatments with Zn 0.5 kg ha^{-1} in 2014 and Se 5 g in 2015. Fertilization with these microelements did not affect the content of P, K, Ca, and Mg.

The treatment with Cu fertilization showed no significant effect on CP, P, K, S, Ca, and Mg contents compared to control.

Micromineral contents in alfalfa biomass

The fertilization of alfalfa with Se and Zn, or these two elements in combination, did not show a significant effect on Mn, Co, and Mo contents in plant biomass, except on the Fe content (Table 6). In the case of Fe, no significant differences between the control treatment and other treatments were found. However, there were significant differences between the 0.5 kg Zn ha^{-1} treatment and the treatments fertilized with 10 g Se ha^{-1} in 2014.

As expected, Cu fertilization significantly increased the Cu content in alfalfa biomass in both years (by 773 and 125%). This treatment did not affect the content of other microelements.

Table 5. Effect of fertilization with selenium, copper, and zinc on CP and macroelement contents in alfalfa biomass (first cut)

	Treatments	CP %	P %	K %	S %	Ca %	Mg %
2014	control	21.25	0.25	1.50	0.36 a	3.38	0.36
	Se 5g	21.90	0.24	1.36	0.30 ab	3.54	0.34
	Se 10g	21.63	0.23	1.28	0.31 ab	3.72	0.37
	Zn 0.5 kg	21.13	0.25	1.30	0.28 bc	3.51	0.31
	Zn 1kg	22.85	0.24	1.62	0.32 ab	3.24	0.27
	Cu 2%	21.79	0.25	1.44	0.30 ab	3.36	0.30
	Zn + Se	21.48	0.25	1.45	0.29 ab	3.44	0.28
2015	control	17.88 bc*	0.22	1.62	0.24 bc	2.28	0.27
	Se 5g	19.58 ab	0.21	1.43	0.32 a	2.07	0.28
	Se 10g	19.90 ab	0.22	1.43	0.27 ac	2.50	0.27
	Zn 0.5 kg	19.02 ab	0.23	1.41	0.25 ac	2.47	0.26
	Zn 1kg	19.81 ab	0.21	1.27	0.27 ab	2.22	0.26
	Cu 2%	19.54 ab	0.22	1.32	0.23 bc	2.48	0.26
	Zn + Se	20.52 a	0.21	1.49	0.20 cd	2.52	0.28
	Annual average	average					
	2014	21.72 A**	0.24 A	1.42 A	0.31 A	3.46 A	0.32 A
	2015	19.46 B	0.22 B	1.42 A	0.25 B	2.36 B	0.27 B

*small letters represent differences between treatments within one year
**capital letters represent differences between years

Table 6. Effect of fertilization with selenium, copper, and zinc on microelement contents in alfalfa biomass (first cut)

	Treatments	Cu mg kg^{-1}	Fe mg kg^{-1}	Mn mg kg^{-1}	Co mg kg^{-1}	Mo mg kg^{-1}
2014	control	6.41 b*	417 ab	54.65	0.29	1.02
	Se 5g	7.43 b	407 ab	57.22	0.27	0.86
	Se 10g	8.27 b	353 bc	59.01	0.29	0.78
	Zn 0.5 kg	7.37 b	591 a	61.56	0.37	0.78
	Zn 1kg	9.63 b	493 ab	56.21	0.38	0.86
	Cu 2%	55.97 a	491 ab	58.00	0.28	0.72
	Zn + Se	8.02 b	336 bc	54.76	0.26	0.88
2015	control	7.19 b	113	26.17	0.06	0.87
	Se 5g	7.28 b	104	27.30	0.05	0.48
	Se 10g	7.38 b	122	26.71	0.05	0.70
	Zn 0.5 kg	7.26 b	129	26.13	0.15	0.59
	Zn 1kg	8.03 b	112	25.54	0.10	0.51
	Cu 2%	16.20 a	120	29.46	0.18	0.55
	Zn + Se	8.97 b	119	25.45	0.12	0.47
	Annual average	average				
	2014	14.73 A**	441 A	57.35 A	0.31 A	0.84 A
	2015	8.90 B	117 B	26.68 B	0.10 B	0.60 B

*small letters represent differences between treatments within one year
**capital letters represent differences between years

DISCUSSION

Se fertilization

Fertilization with Se did not show a significant effect on alfalfa and dry matter yield in all years of the experiment. The results were in agreement with the previous studies that foliar fertilization with Se did not affect the grain or biomass yields of rice (Fang et al., 2008), maize (Wang et al., 2013), and timothy (Tremblay et al., 2015). In a pot experiment, Owusu-Sekyere et al. (2013) found out that selenium addition had a slightly negative effect on the fresh weights and biomass accumulation of alfalfa. However, several studies have shown that Se fertilization may positively affect the yield of different crops. In the experiment with wheat, Se fertigation and foliar spray increased the grain yield of water-stressed plants due to the enhanced production of osmoprotectants and the increased activity of antioxidant enzymes (Nawaz et al., 2015). Similar results have been achieved in the experiment with maize (Nawaz et al., 2016) grown under water deficit conditions. In our research, in the year with the lower amount of precipitation (2015), Se had a slightly positive effect on dry matter yield in the first cut. Selenium fertilization improved Se uptake by alfalfa. As expected, the higher dose showed better results in both years of the experiment.

The applied dose of Se was linearly correlated with the Se content in alfalfa biomass. In a field experiment with maize, Wang et al. (2013) found out that the Se content in maize grain was highly positively correlated with the rate of Se, both foliar and soil applied. In the study performed using a meta-analysis approach based on 243 experiments (from 1960 to 2014), Ros et al. (2016) found out that fertilization characteristics including formulation, dose, and timing were the driving variables enhancing crop Se uptake. According to the same authors, the highest uptake efficiencies have foliar and selenate based fertilizers.

The applied Se did not significantly affect the content of CP or other macro- and microelements, except in the case of S in 2015. In an experiment conducted by Wang et al. (2013), the contents of other elements than Se in maize grain have not been affected by either soil or foliar application of Se.

Zn fertilization

The positive effect of Zn fertilization on alfalfa yield was established in several studies (Grewal 2001; Ceylan et al., 2009; Grewal, 2010). Our study partly confirms these results, because Zn application slightly increased dry matter yield, but without statistical significance. The reason for this could be the lower doses of Zn and foliar application in our experiment, in comparison to other studies with alfalfa, where Zn was applied through soil and in doses of 4 kg Zn ha^{-1} (Grewal 2001; Grewal 2010) or even higher doses of 40, 80, 120 kg ha^{-1} (Ceylan et al., 2009). Also, Grewal et al. (2001) found out that the same Zn dose had different efficiency in increasing the yield of different alfalfa cultivars. The concentration of DTPA Zn in soil could be one of the factors that limit the efficiency of Zn fertilizers. The measured concentration of DTPA Zn in the soil in our experiment was 3.36 mg kg^{-1} and according to Alloway (2009), it was above the critical concentration of 1.5 mg kg^{-1} used in the interpretation of soil test. In the experiments with maize and wheat, Wang et al. (2012) concluded that the soil and foliar application of Zn did not significantly affect biomass or grain yield, due to the soil with sufficient levels of DTPA Zn (more than 0.48 mg kg^{-1}).

In both years of the experiment, the Zn content was highly correlated with the applied Zn doses. Also, Zn fertilization in combination with Se significantly affected the Zn content in the first year of the experiment. Ceylan et al. (2009) also reported that Zn foliar fertilization increased the Zn content, as well as the CP content, in alfalfa biomass. In our investigation, Zn fertilization improved the CP content, especially in the second year, but without statistical significance. The explanation may be the low fertilization dose, as in the case of biomass yield. Zinc accumulation was almost the same in both years, due to the lower yields achieved in the first year of the experiment and a higher Zn content compared to 2015 with higher yields and a lower Zn content. Zinc fertilization did not significantly affect the contents of other elements.

Se and Zn fertilization

According to Sajedi et al. (2011), in corn production under drought stress, Se fertilization is recommended, but not in combination with other microelements, because of their antagonistic effect on the plant and decreased antioxidant activity. The antagonism between selenate and sulfate is well known (Marschner, 1995); therefore, we expected that the applied zinc sulfate would decrease the selenium uptake by alfalfa. However, in our analysis, fertilization with Se and Zn in combination did not show a negative effect on the yield, Se uptake, or mineral composition of alfalfa. The application of these two elements in combination had the same results as when they were applied in the same dose separately. Also, in drought year 2015, the CP content and ARE for Se were the highest on this treatment.

Cu fertilization

Alfalfa belongs to a group of plants with a high sensitivity to copper deficiency (Gupta et al., 2008), but fertilization with Cu did not show the significant improvement of the other observed elements. Fertilization increased the content of Cu in alfalfa biomass; however, the toxic level was reached in plants, especially in the first year of the experiment. According to Gupta et al. (2008), the content of 10-70 mg Cu kg^{-1} is considered to be toxic to plants and is followed by reduced yield. In a higher concentration than that required by plants, Cu may cause chlorosis, necrosis, and leaf discoloration, and it may inhibit plant growth (Yruela, 2005). The measured concentration of Cu in the first year was even higher than the maximum tolerable level of copper (40 mg Cu kg^{-1}) in feed (Suttle, 2010), and such hay is unusable for livestock nutrition. In the present study, the measured concentration of DTPA Cu in soil was relatively high and, in combination with fertilization, it led to the toxic level in plants.

Effect of year and weather conditions

In our experiment, the production year and weather conditions proved to be significant factors in alfalfa production. Alfalfa was sown in the spring of 2014 and due to the lack of precipitation in the period of germination, the establishment was less successful, which also reflected in the yield in all years of the experiment. In Vojvodina, depending on weather conditions, the dry matter yield of alfalfa ranges from 6.4 t ha^{-1} in a drought year to 25.2 t ha^{-1} in a year with a higher amount of precipitation (Ćupina et al., 2016). Also, Tucak et al. (2014) have determined that yield varies significantly depending on alfalfa cultivars. In comparison with the first year, higher dry matter yields were achieved in the second (9.335 kg ha^{-1}) and third year (11.203 kg ha^{-1}) of the experiment, which represent full harvest years, even though the yields were still lower than reported values. The year 2015 could be considered as a drought year, with a lower amount of precipitation compared to the long-term average, accompanied by high temperatures, while 2016 was more favorable and close to these averages.

There were no statistical differences in the average Se contents measured on all treatments in 2014 and 2015. Considering only the treatments with Se applied, the higher Se content was found in 2014 but higher nutrient accumulation was achieved in the second year of the experiment. This could be explained by higher dry matter yield achieved in the first cut in 2015.

The contents of Zn and the other observed elements were significantly affected by the year i.e. the productivity of alfalfa. In 2015, the lower content of all observed elements was recorded, which might have resulted in higher yields and, therefore, the dilution of elements caused by plant growth.

CONCLUSION

Foliar fertilization with Se, Zn, and Cu could be an important agrotechnical measure for increasing these elements in alfalfa grown in slightly alkaline soil under rainfed conditions. However, Cu fertilization should be used with caution, because a high dose may cause the toxic levels of Cu in animal feed. Fertilization with Se and Zn significantly increased their contents in alfalfa plants and did not have a negative effect on the CP content or other macro and microelements. The fertilization efficiency for Se and Zn was high, especially in the case of applied Se. The contents of Se and Zn in alfalfa biomass are strongly correlated (r=0.99) to the applied doses of Se and Zn, respectively. Foliar fertilization with Se and Zn in combination could be recommended because it increased Se and Zn contents in alfalfa and did not show a negative influence on the yield and the content of the other elements. Also, the production year of alfalfa and weather conditions are important factors in the efficiency of applied Se and Zn fertilizers.

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