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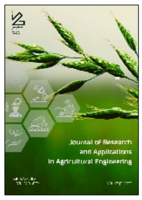

Effect of Soil Biostimulant Application on Protein and Carbohydrate Content in Perennial Ryegrass (*Lolium Perenne* L.)

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One of the elements of modern plant fertilization systems is soil biostimulants. Like fertilizers, they provide plants with nutrients, and thanks to the content of additional substances (e.g. seaweed extracts, amino acids), plant development is affected. The aim of this study is to compare the effect of selected soil biostimulants and traditional P+K+Ca fertilization on changes in the content of crude protein (CP) and water soluble sugars (WSC) in perennial ryegrass (*Lolium perenne* L.) cv. Anna. The research was conducted over a two-year period at the Experimental Station of the Department of Grassland and Natural Landscape Sciences at the Agricultural Experimental Station of Poznan University of Life Sciences. The two-factor experiment was set up in a randomized block design with three replications. The first factor was fertilization with biostimulants (Physio-Mescal G18, PRP-SOL, EM-1 (Effective Microorganisms), EM-1+Ca) and standard mineral fertilizers (P+K+Ca). Nitrogen fertilization (0 and 200 kg·ha^{-1}) was applied as a second factor. The biomass of perennial ryegrass was evaluated for CP and WSC. The experiment showed that the application of Physio-Mescal G 18 and PRP-SOL, as well as EM-1+Ca, resulted in a significant increase in the CP and WSC content in perennial ryegrass compared to the control and the object fertilized with standard P+K+Ca fertilizers. Positive effects on the growth of the plant CP content were noted for two biostimulants: PRP-SOL and Physio Mescal G18. The ryegrass sward from the PRP-SOL-applied object contained 7% more CP compared to the control object, while that from the Physio Mescal G18 object contained 6% more. A very good effect of WSC accumulation was also obtained in the case of the sward from the PRP-SOL and Physio Mescal G18 treatments. In both cases, the determined WSC content was above 141 g·kg^{-1} DM, a difference in content from the control of as much as 6%. In addition, nitrogen fertilization applied at the annual rate of 200 kg·ha^{-1} significantly increased the CP content and decreased the WSC content, which worsened the CP/WSC ratio. The results of the two-year study showed that the incorporation of soil biostimulants, i.e. Physio-Mescal G 18 and PRP-SOL, into fertilizer practice makes it possible to obtain high-quality roughage for livestock.

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1. Introduction

The value of grassland forage is determined by the content of organic components (protein, crude fiber, sugars), minerals and organic matter digestibility. The chemical composition of grasses varies and depends on the species and variety [1], the development stage, the course of meteorological conditions [2] and can be modified by the applied fertilization [3, 4]. Unilateral and long-term fertilization with one or two mineral nutrients can have adverse effects on the soil environment (a decrease in the content of Ca, Mg and other elements, a decrease in pH and depletion of soil microbial life) in addition to the quality of the obtained yields (changes in the botanical composition of the sward and the chemical composition of the plants) [5]. In recent years, so-called soil biostimulants have begun to enter the conventional fertilization of forage crops [6], one of whose tasks is to stimulate and increase soil fertility by activating its underutilized potential inherent in the forms of elements unavailable to plants when the soil pH is too low or too high. There is also growing interest in fertilizers and preparations that have a comprehensive effect on improving soil chemical properties such as the pH, nutrient availability, and increasing the activity of microbial flora in the plant rhizosphere [3, 7]. Biostimulants, soil improvers and conditioners, are designed to provide an improved soil structure, faster mineralization of plowed organic matter, and better assimilation of mineral nutrients, such as phosphorus and potassium deposited in soil mineral reserves that are difficult for plants to access. Biostimulants and fertilizers of this type are produced on the basis of chemically active calcium from marine deposits in France [8]. Calcium in these fertilizers plays a dual role: as a carrier of marine algae extract, aimed at activating microbial flora depleted by crop monocultures, and supplementing micronutrient deficiencies in the soil. The calcium contained in biostimulants is designed to raise the soil pH [3] and reduce the effects of harmful aluminum and manganese ions on the plant root system. Biostimulants and various fertilizers of this nature include: PRP-SOL, Physio-Mescal G 18, EM (Effective Microorganisms) and its various modifications (EM-1, EM-5, EM-A, EM-Farming) as well as Physactiv+1 or Sulfammo 23 N-Process, Soleflor [9, 10]. The effect of fertilizers and biostimulants in field studies is more visible and easier to interpret when testing them on a single plant species. In meadow studies, diploid or tetraploid cultivars of perennial ryegrass are most often selected for this purpose [1, 4, 11, 12]. Perennial ryegrass (*Lolium perenne* L.) is a very popular species of the forage grass group in temperate climates [13, 14]. This species has a high potential for producing good quality forage for livestock. Under grazing conditions, it maintains a high growth rate during the growing season and resistance to trampling and biting. The diploid varieties of this species are more durable and more tolerant of grazing, mowing frequency and weather stresses. Tetraploid varieties have better tillering potential, a faster rate of regrowth after defoliation and a higher feed value [15]. The pasture variety of perennial ryegrass cv. Anna is an early diploid variety (K > 45), characterized by high spring growth energy and a fast regrowth rate after defoliation in addition to a high yield potential [16]. Having a high yield potential, it requires appropriate fertilization to maximize its productivity and nutritive value. The research hypothesis was that the application of soil biostimulants would modify the nutrient composition of a perennial ryegrass sward compared to traditional fertilization with NPK mineral fertilizers and the control.

The purpose of the study is to evaluate the effect of fertilization with soil biostimulants: Physio-Mescal G 18, PRP-SOL, microbial preparation EM (Effective Microorganisms) and standard NPK mineral fertilizers on the crude protein (CP) content and water soluble carbohydrates (WSC) in perennial ryegrass (*Lolium perenne* L.) cv. Anna in field sowing.

2. Material and methods

2.1 Experimental field

The research was conducted over a two-year period at the Experimental Station of the Department of Grassland and Natural Landscape Sciences at the Agricultural Experimental Station of Poznan University of Life Sciences (52°43' N, 16°30' E). The soil on which the experiment was established was light loamy sand with a humus horizon thickness of more than 30 cm belonging to soil valuation class IIIb. The soil was characterized by a 16% share of floatable parts, a humus content of 1.24%, a neutral pH (6.7) and a high content of phosphorus (181 g P_2O_5 in kg^{-1} DM of soil), a medium content of potassium (229 g K_2O in kg^{-1} DM of soil) and a low content of magnesium (45.1 g MgO in kg^{-1} DM of soil). According to the international WRB classification the soil was classified as Albic Luvisols and according to Soil Taxonomy as Typic Hapludalfs in terms of grain size as loamy sand [17]. The sowing of seeds of perennial ryegrass (*Lolium perenne* L.) cv. Anna in experimental plots was carried out manually in autumn in the year preceding the study at the rate of 30.0 $kg \cdot ha^{-1}$. The experiment was established by the randomized block method in three repetitions in plots of 7.5 m^2 (1.5 m x 5.0 m). The distance between the plots was 0.5 m in order to reduce the boundary effect of the fertilizers. Two experimental factors were applied: the first factor was fertilization with biostimulants (Physio-Mescal G18, PRP-SOL, EM-1 (Effective Microorganisms), EM-1+Ca and P+K+Ca) and standard P+K+Ca mineral fertilizers, and as the second factor nitrogen fertilization (0 and 200 kg $N \cdot ha^{-1}$ in split doses of 50 kg $N \cdot ha^{-1}$ each per harvest) was applied. In the experiment (standard fertilizers), phosphorus was applied in the form of granular triple superphosphate 46% P_2O_5, potassium in the form of potassium salt 60% K_2O, as well as calcium in the form of fertilizer lime 48% CaO. Ammonium nitrate 34% N was used for nitrogen fertilization in each variant. The control consisted of plots without the application of fertilizers and biostimulants. In the spring before the start of vegetation, the following doses of soil biostimulants and mineral fertilizers were applied in standard fertilization:

- Physio-Mescal G 18 (18% P_2O_5, 65% $CaCO_3$, 5% MgO and patented Physio+ brown algae extract) - dose of 450 $kg \cdot ha^{-1}$. This introduced into the soil: 81 $kg \cdot ha^{-1}$ P_2O_5, 292.5 $kg \cdot ha^{-1}$ $CaCO_3$ and 22.5 $kg \cdot ha^{-1}$MgO.
- PRP SOL (30% CaO, 8% MgO, 3.5% Na and 3-5% prefixes, containing 48 trace elements needed for proper plant growth and development) - dose of 200 $kg \cdot ha^{-1}$. Introduced into the soil in this way: 60 $kg \cdot ha^{-1}$ CaO, 16 $kg \cdot ha^{-1}$ MgO, 7 $kg \cdot ha^{-1}$ Na, 6-10 $kg \cdot ha^{-1}$ micronutrient elements.
- EM-1 - 1.0 liter of EM-1 + 100 liters of water · per ha^{-1},
- EM-1+Ca - 1.0 liter of EM-1 + 100 liters of water per ha^{-1} + 100 $kg \cdot ha^{-1}$ $CaCO_3$,
- P+K+Ca - standard fertilization applied in doses: triple superphosphate 174 $kg \cdot ha^{-1}$ + potassium salt 133 $kg \cdot ha^{-1}$ + 416 $kg \cdot ha^{-1}$ carbonate lime. The following were introduced into the soil: 80 $kg \cdot ha^{-1}$ P_2O_5 + 80 $kg \cdot ha^{-1}$ K_2O + 200 $kg \cdot ha^{-1}$ $CaCO_3$.

2.2 Laboratory assays

The perennial ryegrass (*Lolium perenne* L.) was used for hay, harvesting four times during the growing season. Each year, a 1 kg sample of biomass was taken for analysis during each harvest. In the samples of the plant material, after they were dried and ground, the following were evaluated: CP, the content of which was determined from total nitrogen using the Kjeldahl method (Tecator Kjeltec 2300, Foss, Hilleroed, Denmark), and WSC using the method of Dubois et al. [18]. Based on the determined amount of WSC and CP, the WSC/CP ratios were calculated. Due to the low variability of the results in the years of the study, the tabulated data are presented as averages of the two years of the study.

2.3 Statistical analysis

Statistical processing of the results was performed using Statistica v. 6.0. The Shapiro-Wilk test and the Levene test were used to statistically determine the normality and variance homogeneity of the data prior to the analysis. The model included the fixed effects of biostimulant application (A) and nitrogen dose (B). A two-way analysis of variance (ANOVA) was then used to examine the differences between the treatments. The significance of the effect of the studied experimental factors was verified using the Tukey test at the significance level of $\alpha = 0.05$.

2.4 Meteorological conditions

The first year of the study was characterized by slightly higher average temperatures in May, June, July and October than the second year. Compared to the multi-year period, the second year of the study was characterized by temperatures higher by 1.5° C in March, 3.9° C in April, 1.7° C in July, 2.3° C in August and 2.4° C in September, respectively. The total precipitation compared to the multi-year period was lower in the first year of the study by 12.9 mm·m^2 and in the second year by as much as 147.0 mm·m^2. The second year of the study was characterized by a total precipitation of only 446.7 mm·m^2. In the second year of the study, in none of the months during the growing season did the precipitation sum exceed 90.0 mm·m^2, which was recorded in the first year of the study: in April (120.7 mm·m^2) and in August (171.5 mm·m^2) (Fig. 1).

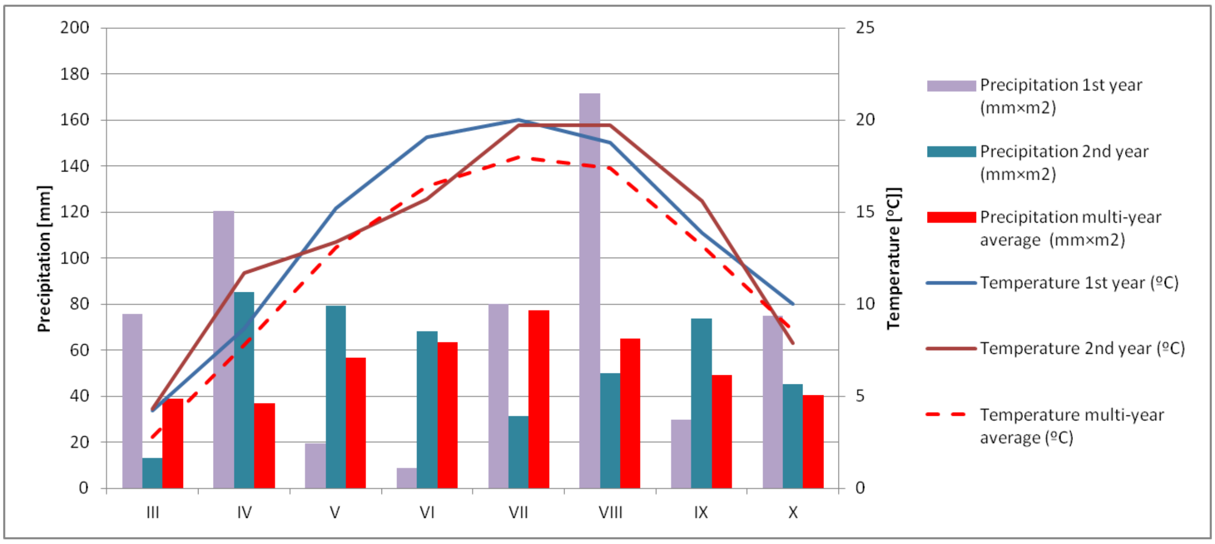


Fig. 1. Meteorological conditions during growing season in years of conducted research. Source: authors' own compilation based on data from meteorological station in RGD Brody.

3. Results

3.1. Total protein (CP)

One of the most important components affecting the feed value of grassland forage is the CP content. In grasses intended for grazing, its optimal content should be 160-180 g·kg^{-1} DM and in forage intended for silage 140-160 g·kg^{-1} DM [19]. It is assumed that the content of CP above 220 g·kg^{-1} DM can be a signal indicating a dangerous concentration of harmful forms of nitrogen for animals [5].

The content of this component in perennial ryegrass varied and depended on both the nitrogen rate and the use of soil biostimulants (Table 1).

On average, the plants from the plots fertilized with nitrogen at 200 kg·ha^{-1}, on all the harvest dates, contained significantly more CP than the plants from the plots not fertilized with nitrogen. It was shown that the CP content of perennial ryegrass in the variant with nitrogen application ranged from 83.3 g·kg^{-1} DM (control object in the 4th harvest) to 131.47 g·kg^{-1} DM (Physio Mescal G18 in the 2nd harvest). In comparison, in the variant without nitrogen application, the content of CP ranged from 58.43 g·kg^{-1} DM (control in 4th harvest) to 109.83 g·kg^{-1} DM (Physio Mescal G18 in 2nd harvest) (Fig. 2). The difference in the CP content of the perennial ryegrass sward based on the harvest averages between the variant with and without N application was almost 26% (22.9 g·kg^{-1} DM).

Regardless of the nitrogen fertilization rate, on average the highest content of CP was found in the perennial ryegrass from the plots fertilized with P+K+Ca fertilizers (104.3 kg^{-1} DM) and from the plots where the biostimulants were applied: Physio Mescal G18 (104.0 kg^{1} DM) and PRP-SOL (103.1 kg^{-1} DM) (Table 1).

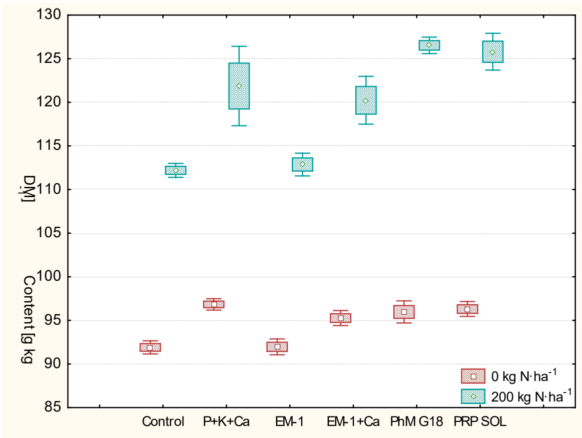
Table 1. Effect of soil biostimulant application and N fertilization rate on CP content in perennial ryegrass - average content from years of study (g·kg^{-1} DM)

Factor studied	Research treatments	1st harvest	2nd harvest	3rd harvest	4th harvest	Mean from all harvests
Type of biostimulant (A)	Control	102.1a±11.1	110.9a±8.7	93.3a±10.1	70.9a±13.8	94.3a±10.9
	P+K+Ca	109.4bc±14.0	119.4c±10.7	99.8b±12.4	88.7c±13.2	104.3c±12.4
	EM-1	102.4a±11.5	112.2ab±9.1	93.9a±9.3	71.7a±14.6	95.1a±10.9
	EM-1+Ca	107.8b±13.8	116.5bc±9.4	99.1b±11.5	75.1a±15.4	99.6b±12.4
	Physio Mescal G18	111.3c±16.8	120.7c±11.9	101.8b±11.5	82.2b±20.7	104.0c±15.2
	PRP-SOL	111.1c±16.2	119.8c±11.6	101.0b±10.4	80.6b±18.2	103.1c±14.0
Nitrogen dose (B)	0	94.7a±2.2	107.5a±2.9	88.4a±3.6	63.8a±6.8	88.6a±3.2
	200	119.9b±6.2	125.7b±5.9	107.9b±4.5	92.6b±7.9	111.5b±5.7
	Mean for treatments	107.3C±13.6	116.6D±10.3	98.2B±10.6	78.2A±16.3	
			Interactions			
	A	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
	B	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
	AxB	*<0.001*	*0.112*	*0.388*	*0.003*	*<0.001*

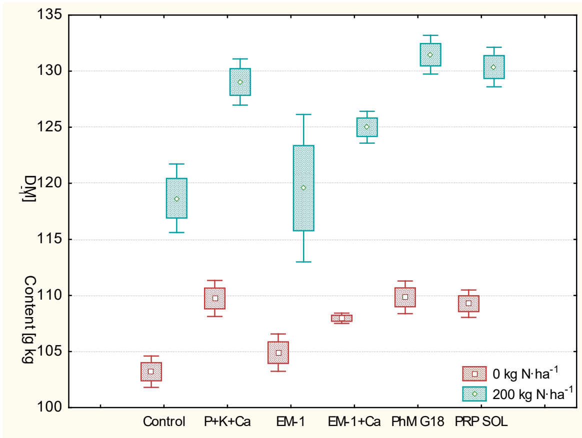
Means with same letter do not differ significantly at $p < 0.05$ in Tukey's HSD test.

In analyzing the average content of CP from the harvests in the variant without N, it can be concluded that the highest content of this component was determined in the perennial ryegrass sward after the application of traditional P+K+Ca fertilization (93.06 g·kg^{-1}DM), and the difference from the absolute control was 10% (8.6 g·kg^{-1} DM). Positive effects on the growth in the plant CP content were also noted for two biostimulants: PRP-SOL and Physio Mescal G18. The ryegrass sward from the PRP-SOL-applied object contained 7% (6.01 g·kg^{-1} DM) more CP compared to the control object, while that from the Physio Mescal G18 object contained 6% more (5.73 g·kg^{-1} DM). Both treatments where these biostimulants were applied were not much inferior in this respect to the sward of the object with the P+K+Ca application. Based on the analysis of the average CP content of the harvests in the variant with N application, it turned out that the sward of the PRP SOL object had a similar content of this component and in the case of Physio Mescal G18 it was even higher by 1.7% compared to the object fertilized with traditional P+K+Ca fertilizers. The difference in the CP content between the P+K+Ca object and the control was 11% (11.5 g·kg^{-1} DM). It can be concluded that in the case of this component, the applied nitrogen did not adversely affect the performance of the biostimulants, and even better effects of Physio Mescal G18 were found compared to the P+K+Ca treatments. An interesting reaction occurred in the treatments with the EM-1+Ca application. Under the influence of the additional application of Ca on this object, a higher CP concentration in perennial ryegrass could be observed than in the treatments where only the microbial preparation EM-1 was applied. In the variant without N, this difference was 3.6% and with N application, the difference was 5.6% (Fig. 2).

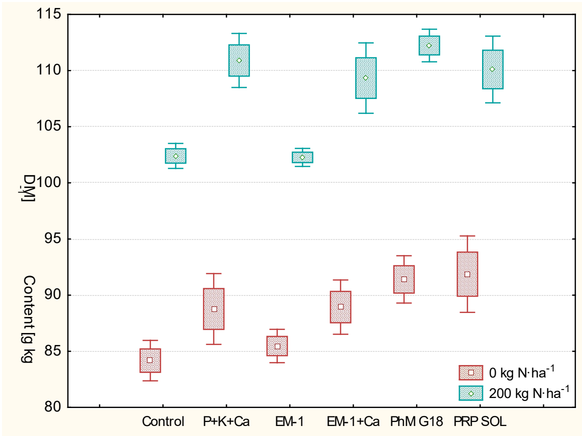
In addition to fertilization, there was a significant effect of the harvest number on the CP content of the perennial ryegrass sward. The highest CP content was found in the plants from the 2nd harvest (average 116.6 g·kg^{-1} DM) and the lowest in the 4th harvest (78.16g·kg^{-1} DM). The highest content of the studied component in the plants from the 2nd harvest was found on the object with the application of Physio Mescal G18 (131.4 g·kg^{-1} DM) and PRP-SOL (130.37 g·kg^{-1} DM). The differences in the CP content between the object with the EM-1 application and the control were small; this was evident in both variants, without N and with its application.



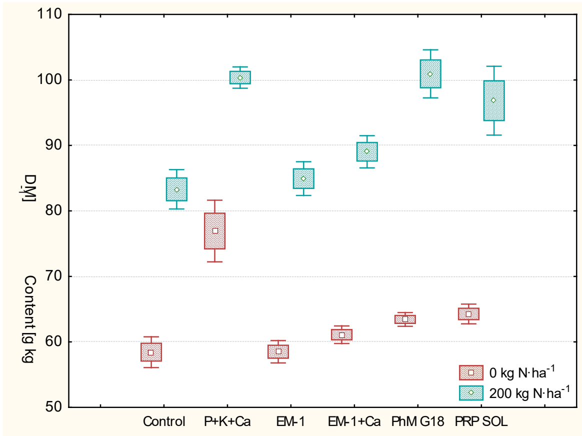
a) 1st harvest



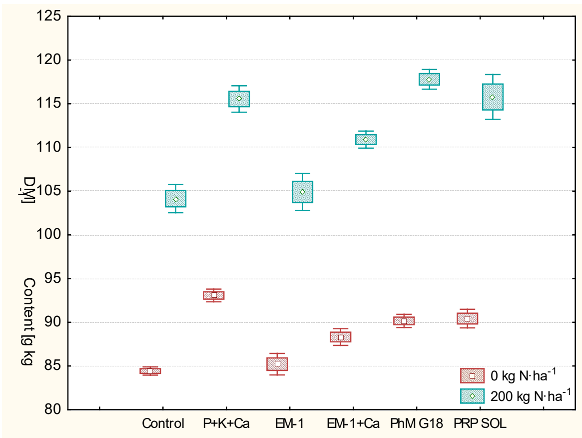
b) 2nd harvest



c) 3rd harvest

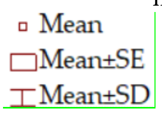


d) 4th harvest



e) mean from all harvests

Fig. 2. CP content in *Lolium perenne* L. cv. Anna biomass in following harvests.



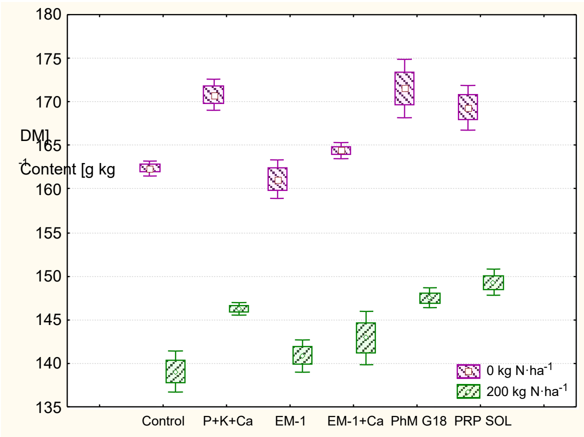
3.2. Soluble Sugars (WSC)

Soluble sugars is another component that plays a very important role in the evaluation of the feed value of forage grasses. They influence the improvement in forage palatability, energy content and the balance of the protein-to-energy ratio. A higher content of this component affects the proper course of the silage process. The WSC content depended on both the nitrogen fertilization and soil biostimulants used (Table 2). The differences in the WSC content in the sward between the different treatments where the soil biostimulants were applied were statistically significant. The highest WSC content (above 187.00 g·kg^{-1} DM) was characterized by the perennial ryegrass sward in the second harvest in the variant without nitrogen fertilization in the treatments where Physio Mescal G-18 and traditional P+K+Ca fertilization were applied. Based on the average results of the content of WSC from the harvests in the variant without N, it was found that the perennial ryegrass sward had the highest content of this component after the application of Physio Mescal G18 (164.58 g·kg^{-1} DM) and standard P+K+Ca fertilization (164.08 g·kg^{-1} DM), and the difference in the content compared to the control was above 6% in both cases. Also a high content of this component, above 162 g·kg^{-1} DM, was found in the sward on the plots with PRP-SOL application, which also indicates the positive effect of this biostimulant on the accumulation of WSC in plants. The sward from the absolute control and the facility with the EM-1 application did not differ in the content of this component between them. In both cases, the WSC content was found to be at the level of 154 g·kg^{-1} DM. In the variant with N fertilization, the content of this component was determined in the sward, ranging from 112.13 g·kg^{-1} DM to 157.13 g·kg^{-1} DM (Fig. 3). The least amount of WSC was recorded in the sward of the variant with N application on the EM-1 plots in the 4th harvest (112.13 g·kg^{-1} DM) and the highest WSC content was determined in the 2nd harvest on the treatments after the application of the soil biostimulant Physio Mescal G18 (157.13 g·kg^{-1} DM). Analysis of the average results obtained from the harvests in the variant with nitrogen application showed that the sward from the object fertilized with P+K+Ca had the highest WSC content (142.43 g·kg^{-1} DM). The difference in the content of this component between this object and the control was 7% (9.62 g·kg^{-1} DM). A very good effect of WSC accumulation was also obtained in the case of the sward from the PRP-SOL and Physio Mescal G18 treatments. In both cases, the determined WSC content was above 141 g·kg^{-1} DM, a difference in content from the control of as much as 6%. The applied nitrogen fertilization in this variant reduced the content of WSC in the sward for all the test treatments. The difference in the WSC content of the perennial ryegrass sward between the variant with and without N application was 15% (21.5 g·kg^{-1} DM). As in the case of the variant without N application, the differences in the content of this component between the EM-1 object and the control were small (0.63 g·kg^{-1} DM). However, it was noticeable that under the influence of the Ca application in the object with EM-1 in the variant with N fertilization, there was an increase in the WSC content in the plants by 3.8% (5.14 g·kg^{-1} DM) compared to the control and in the variant without N there was an increase of only 1.8% (2.9 g·kg^{-1} DM).

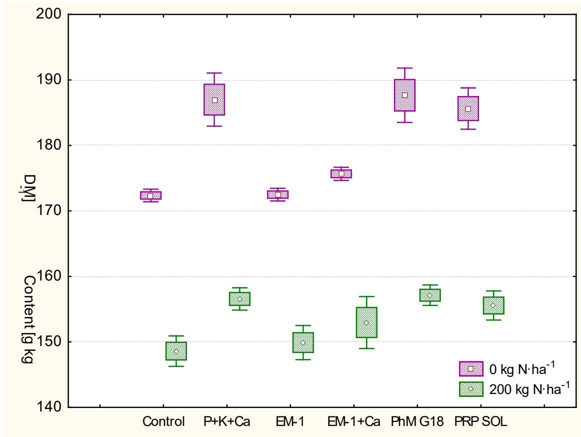
Table 2. Effect of soil biostimulant application and N fertilization rate on WSC content in perennial ryegrass - average content from years of study (g·kg^{-1} DM)

Factor studied	Research treatments	1st harvest	2nd harvest	3rd harvest	4th harvest	Mean from all harvests
Type of biostimulant (A)	Control	150.7a±12.9	160.5a±13.1	141.8a±11.8	122.6a±11.2	143.9a±12.1
	P+K+Ca	158.5b±13.5	171.8b±16.9	151.7c±8.2	131.1c±9.8	153.3c±11.9
	EM-1	151.0a±11.2	161.2a±12.5	142.0a±12.3	121.8a±11.0	144.0a±11.6
	EM-1+Ca	153,7a±11.9	164,3a±12.7	148,4b±10.0	125,2ab±9.8	147,9b±11.0
	Physio Mescal G18	159,5b±13.4	172,4b±17.0	151,7c±9.7	128,2bc±11.6	153,0c±12.8
	PRP-SOL	159.3b±11.1	170.6b±16.6	151.3c±8.8	127.7bc±9.8	152.2c±11.5
Nitrogen dose (B)	0	166.6b±4.6	180.1b±7.3	157.0b±3.3	157.0b±3.7	157.0b±4.4
	200	144.3a±4.1	153.5a±4.0	138.7a±6.0	138.7a±4.5	138.7a±4.2
	Mean for treatments	155.5C±12.1	166.8D±14.7	147.8B±10.4	126.1A±10.3	
		Interactions				
	A	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
	B	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
	AxB	*0.267*	*0.017*	*<0.001*	*0.805*	*0.368*

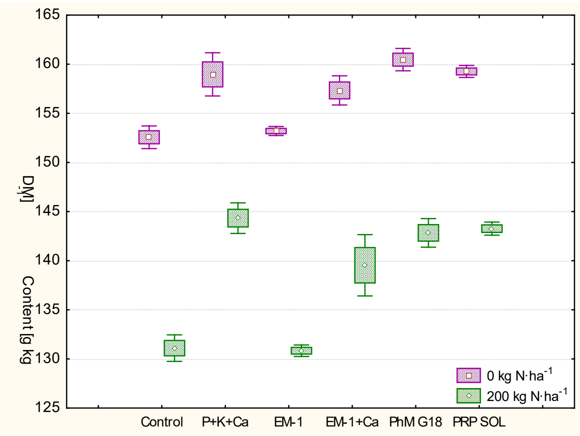
Means with same letter do not differ significantly at $p < 0.05$ in Tukey's HSD test.



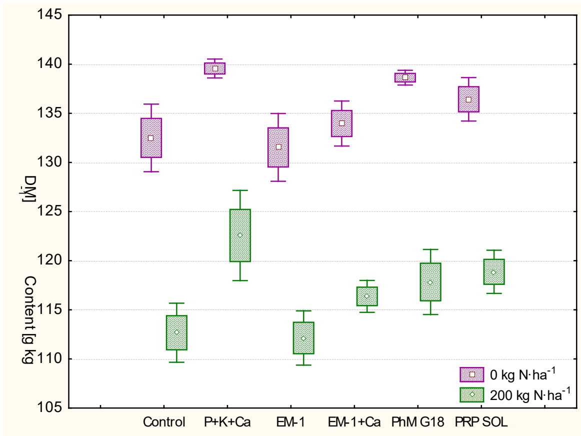
1. 1st harvest



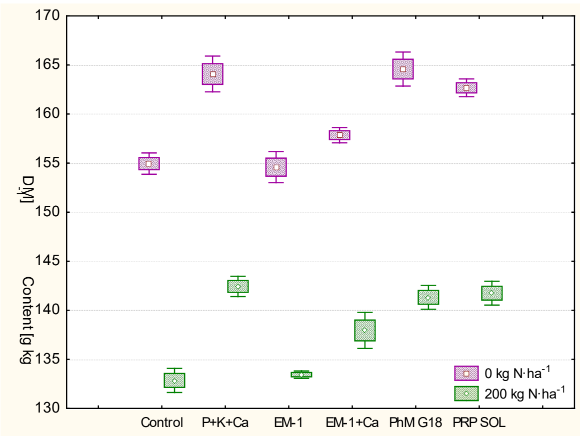
2. 2nd harvest



3. 3rd harvest

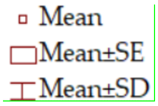


4. 4th harvest



5. mean from all harvests

Fig. 3. WSC content in *Lolium perenne* L. cv. Anna biomass in following harvests.



3.3. Sugar-protein ratio (WSC/CP)

The WSC/CP ratio depended on both nitrogen fertilization and the soil biostimulants used (Table 3). Significant differences in the WSC/CP ratio were noted between the applied fertilizer variants only on the last harvest date, when the perennial ryegrass sward fertilized with P+K+Ca had a significantly lower WSC/CP ratio than the sward of this species from the other fertilizer variants. Such differences were not found in the first, second and third harvests. In all the harvests, the WSC/CP ratio was significantly affected by N fertilization. The application of an N rate of 200 kg·ha^{-1} negatively affected the WSC/CP ratio, significantly reducing its value on average from 1.84 to 1.25 (Table 3). There were also significant differences in the WSC/CP ratio between the swaths. The WSC/CP ratio was the highest in the 4th forage harvested (1.70), and the lowest in the forage from the 1st (1.48) and 2nd harvest (1.45).

Table 3. Effect of soil biostimulant application and N fertilization rate on CP/WSC ratio in perennial ryegrass - average content from years of study (g·kg^{-1} DM)

Factor studied	Research treatments	1st harvest	2nd harvest	3rd harvest	4th harvest	Mean from all harvests
Type of biostimulant (A)	Control	1.50a±0.04	1.46a±0.03	1.55a±0.04	1.81b±0.15	1.58c±0.04
	P+K+Ca	1.48a±0.04	1.46a±0.03	1.55a±0.03	1.52a±0.09	1.50c±0.04
	EM-1	1.50a±0.03	1.45a±0.03	1.54a±0.04	1.79b±0.19	1.57bc±0.05
	EM-1+Ca	1.46a±0.03	1.43a±0.06	1.52a±0.07	1.75b±0.17	1.54abc±0.03
	Physio Mescal G18	1.48a±0.03	1.45a±0.06	1.51a±0.02	1.68b±0.08	1.53ab±0.04
	PRP-SOL	1.47a±0.04	1.45a±0.02	1.52a±0.02	1.68b±0.06	1.53ab±0.02
Nitrogen dose (B)	0	1.76b±0.03	1.68b±0.04	1.78b±0.05	2.14b±0.17	1.84b±0.04
	200	1.20a±0.27	1.22a±0.27	1.29a±0.28	1.27a±0.26	1.25a±0.28
	Mean for treatments	1.48A±0.28	1.45A±0.23	1.53AB±0.25	1.70B±0.46	
			Interactions			
	A	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
	B	*<0.001*	*<0.001*	*<0.001*	*<0.001*	*<0.001*
	AxB	*<0.001*	*<0.001*	*<0.001*	*0.003*	*<0.001*

Means with same letter do not differ significantly at $p < 0.05$ in Tukey's HSD test.

4. Discussion

The results of the study indicate that the content of the tested parameters in the biomass of the perennial ryegrass was influenced by both the experimental factors, which have important practical significance. Among the tested biostimulants effective microorganisms were used, which were also applied in combination with Ca. The results of previous studies testing the EM-1 preparations are not unequivocally positive. The described effects are either very large or small [20, 21], and it is often the case that the use of these preparations does not have the expected effects [22, 23]. In our study, the applied EM-1 preparations had no significant effect on the content of the tested components, the content of which was at the same level as on the control. The addition of Ca caused a significant increase in the content of CP and WSC both in relation to the control and the variant on which the EM-1 preparation was applied. The other soil biostimulants were Physio-Mescal G 18 and PRP-SOL. Physio-Mescal G 18 is a biostimulant that improves the structure and stabilizes the soil reaction, stimulates the enzymatic activity of soil microorganisms, thus influencing better absorption of nutrients by plants. In addition, it positively influences the development of the root system, increasing the surface extent of the uptake of mineral nutrients from the soil by plants [8]. PRP-SOL is a mineral biostimulant that supports the biological activity of the soil, produced on the basis of calcium carbonate, which is additionally enriched with trace elements. Calcium, microelements and the seaweed extract contained in this fertilizer increase the uptake of phosphorus, potassium and trace elements by plants [24, 25]. The effect of both biostimulants on the content of the evaluated parameters was the same. Both contributed to a significant rise in the content of CP and WSC in relation to their content in perennial ryegrass from the other fertilizer treatments. The effectiveness of both biostimulants was mainly due to the presence of calcium in them. Along with the application of Physio-Mescal G 18, nearly 300 kg·ha^{-1} $CaCO_3$ was introduced annually and 60 kg·ha^{-1} CaO was introduced when PRP-SOL was applied. In addition, phosphorus, magnesium sodium and other trace elements were supplied, which had a beneficial effect on the content of the studied components in perennial ryegrass. According to a study conducted by Zielewicz et al. [3, 26], the application of the

Physioactiv biostimulant containing calcium carbonate (76% $CaCO_3$) reduced the negative effect of long-term nitrogen fertilization and kept the soil pH constant. The effect of nitrogen fertilization on the content of CP in plants is known. The applied nitrogen dose of 200 kg·ha^{-1} had a significant effect on increasing the content of this component in the forage. The results also confirm the studies of Ihtisham et al. [27] and Olszewska [4, 11], who noted an increase in the CP concentration in perennial ryegrass forage in response to nitrogen fertilization. According to the cited authors, this growth was due to an increased rate of photosynthesis and the fact that the level of CP content depends on the intensity of this process in the plants. Also, Jankowska [28] in her study confirms that incrementing the doses of nitrogen fertilization raised the content of CP in the forage and decreased the content of WSC in it. In the described studies, the content of CP varied significantly between the harvests. The CP content of the ryegrass biomass was the highest in the second harvest, significantly lower than in the 1st harvest, and it significantly decreased sequentially in the 3rd and 4th harvests. The variability in the CP content between harvests was also observed by Olszewska [4], with the difference that the content in the biomass of the tested ryegrass varieties grew significantly between the first and subsequent harvests by about 15%, 24% and 41%, respectively. According to Schlegel et al. [29], the CP content of grass biomass is lower in the first harvest because the grasses in this harvest occur only at the generative stage. The WSC content of grass biomass is significantly affected by N fertilization. In our study, the WSC accumulation was lower in the perennial ryegrass plants fertilized with nitrogen at the rate of 200 kg N·ha^{-1}. Similar results were presented by Olszewska [4] and Chesney et al. [30], among others. A pronounced decrease in the content of WSC under the influence of high doses of nitrogen some authors explained by a large accumulation of nitrogenous substances in the plants, for the construction of which carbohydrates are primarily consumed. The content of WSC also depended on the harvest date. The highest WSC content was characterized by the plants in the 2nd harvest, significantly lower in the first and third, and the lowest in the last - the fourth. Similar changes in the WSC content in different harvests of perennial ryegrass were observed in their studies by Olszewska [4], Truba et al. [12] and Purwin et al. [1]. In our study, the WSC/CP ratio was in the range of 1.2-1.50, which is considered optimal in rations for ruminants [31]. On average, the WSC/CP ratio was higher in the N-unfertilized forages. Significant differences were also found in the WSC/CP ratio between the swaths. The WSC/CP ratio was the highest in the green forage harvested in the 4th, and the lowest in the green forage from the 1st and 2nd swaths. In conclusion, the experiment demonstrated that some biostimulants can be a complement and even for a short time an alternative to traditional mineral fertilization of P and K, especially in fertilization systems on organic farms. Compared to traditional mineral fertilizers, they can help more in maintaining and modifying the soil's biological potential and fertility, especially where manure or other natural and organic fertilizers are not used.

The literature on the use of soil biostimulants on the nutritional value of the sward is not very extensive in databases. Many of the studies in this field focus on the effect of such biostimulants on the plant yield or soil microbial activity. Sosnowski et al. [32] observed a positive effect of soil biostimulants on the nutritive value of various forage grass species. Sulewska et al. [25] report that several microbial preparations are already used in agriculture and horticulture with promising effects in increasing soil fertility. The positive effects of biostimulants and soil improvers in cultivated plants were reported in their studies by Trawczyński and Bogdanowicz [33] and Sosnowski and Jankowski [34]. Our study also focused on the properties of the microbial biostimulant EM-1, which has received both favorable and unfavorable reviews depending on the scientific studies. In our study, the application of the soil biostimulant EM-1 in perennial ryegrass cultivation did not have the intended effect and the obtained results were similar to those in the absolute control without the application of biostimulants and mineral fertilizers. Potential limitations to the wider use of such biostimulants will be their price, which depends on the policies of their producers. Higher prices compared to the cost of purchasing traditional mineral fertilizers may reduce farmers' interest in this type of agricultural option. A second limitation for biostimulants may be their low availability at agricultural supply points. Limitations to the use of biostimulants can also include weather conditions and the choice of the right time for their application as some biostimulants, especially those containing different types of bacterial inoculants in their composition, require application directly to moist soil or application during rainfall. The occurrence of drought following the application of such preparations reduces the effect or completely prevents the multiplication of the introduced bacteria and their positive effects on the soil and plants. Weather conditions are extremely important when applying preparations containing bacterial strains as it is recommended that they be applied during rainy weather and at the appropriate air temperature. Probably as a result of errors in their application, there are reports of unsatisfactory effects after the application of microbial biostimulants in field crops [35,36]. Another factor limiting the wider use of biostimulants is the farmer's knowledge of their availability on the market and how they can be applied to specific production lines on the farm. New biostimulants that have recently appeared on the market (BlueN) are plant biostimulants containing bacteria that inhabit aboveground plant parts and roots increasing nitrogen fixation by plants also from other botanical families (not only legumes). A new research direction initiated by the researchers should be to combine the use of soil biostimulants and new plant biostimulants in agriculture. Such activities should provide an answer to the extent to which they will be useful in reducing the use of fertilization with traditional mineral fertilizers, particularly nitrogen fertilizers. The use of biostimulants is not limited to agricultural applications for improving soil microflora or crop growth and yield. A direction that will be more widely developed in further research is the application of biostimulants in carbon farming and their wider use to improve plant health and enrich soil microbiology under conditions of plant cultivation in long-term cereal monocultures. A future direction of research is also the possibility of using biostimulants in organic farms and in the reclamation of degraded soils in addition to the elimination of water contamination by chemical and microbiological pollutants [37,38]. Also, a future direction will be the use of such microbiological preparations in animal husbandry as factors improving the sanitary conditions of animals and accelerating the fermentation of manure, and seaweed extracts as feed additives improving animal health [39,40].

5. Conclusions

1. Among the applied soil biostimulants, only two, i.e. Physio-Mescal G 18 and PRP-SOL, had an effect on the content of the evaluated nutrients. Both biostimulants caused a significant increase in the CP content and WSC compared to the content of these components in the perennial ryegrass sward from the other fertilizer sites.
2. Positive effects on the growth of the plant CP content were noted for two biostimulants: PRP-SOL and Physio Mescal G18. The ryegrass sward from the PRP-SOL-applied object contained 7% more CP compared to the control object, while that from the Physio Mescal G18 object contained 6% more. A very good WSC accumulation effect was also obtained in the sward after the application of PRP-SOL and Physio Mescal G18. In both cases, the determined WSC content was as much as 6% higher compared to the control.
3. The EM-1 preparations had no significant effect on the content of the tested components, the content of which was at the same level as determined in the perennial ryegrass sward on the control. The application of Ca to the applied EM-1 preparation caused a significant increase in the content of CP and WSC both in relation to the control and the variant on which only the EM-1 preparation was applied.
4. The results of the two-year study showed that nitrogen fertilization applied at an annual rate of 200 kg·ha^{-1} significantly increased the CP content and decreased the WSC content, which worsened the WSC/CP ratio.
5. The results obtained on the influence of soil biostimulants on the content of basic nutrients in plants, i.e. protein (CP) and soluble sugars (WSC), indicate that this is an area of research which should be continued and expanded in the future to include an analysis of the influence of these biostimulants on other qualitative characteristics of the sward, such as NDF, the ADF content, digestibility and the mineral composition.
6. Research into new possibilities for the use of biostimulants will continue and be developed. New directions for their use in agriculture and environmental protection continue to be sought. Limitations to the wider use of biostimulants will be their price, availability on the market and their effectiveness and competitiveness against mineral fertilizers. Timely application under suitable weather conditions is very important for their high efficiency.

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