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Yield Performance and Content of Selected Organic Compounds in *Trifolium pratense* Treated with Various Biostimulants against the Background of Nitrogen Fertilisation

A. Godlewska, G.A. Ciepiela

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ABSTRACT

The present work is an attempt to assess the effect of biostimulants of various origins and nitrogen regime on yield performance, chlorophyll content, protein content and soluble carbohydrate in red clover. A field experiment was arranged as a randomized subblock design (split-plot) in Poland in August 2013. The following factors were examined: type of biostimulant; nitrogen application rate: 0 (control) and 30 kg· ha^{-1}. Biostimulants significantly increased red clover biomass yields. All the examined biostimulants contributed to an increase in the amount of organic components in red clover dry matter. Nitrogen applied at the rate of 30 kg· ha^{-1} significantly increased red clover yield, chlorophyll content in red clover leaves and protein compounds but it also significantly reduced soluble carbohydrates in the dry matter of test plants.

Key words: Chlorophyll, Fertilization, Protein compounds, Soluble carbohydrates.

INTRODUCTION

Biostimulants are products of natural origin (Kocira *et al.,* 2018) containing biologically active natural compounds which, when used in agricultural crops, are safe for the environment (Tuhy *et al*., 2013). As a result, the application of such products to enhance crop plant production on a global scale, particularly in sustainable agriculture, is increasing by the year as new technologies are sought to reduce reliance on expensive mineral fertilisers and plant protection agents which have a negative effect on the soil environment and simultaneously obtain healthy food of high quality. The literature on the subject includes works reporting diverse effects of biostimulants on yield performance and quality, increase in nutrient availability for plants, water holding capacity, antioxidant content and chlorophyll production in plants (Khan *et al*., 2009; Godlewska and Ciepiela, 2016a). Active compounds present in these products are effective even at low concentrations so they can be applied at very low rates. However, there is still lack of research which comprehensively describes response of many plant species to growth regulators. Thus, an attempt was made to assess the effect of biostimulants of various origins and nitrogen regime on yield performance, chlorophyll content, protein content and soluble carbohydrate in red clover.

MATERIALS AND METHODS

A field experiment was arranged as a randomized subblock design (split-plot) with three replicates at the Siedlce Experimental Unit of the University of Natural Sciences and Humanities in August 2013. The plot area was 10 m^2. According to the Polish soil classification system (Systematics of Polish Soils 2011), the soil used in the experiment was the culture soils with the hortisole type, developed from loamy sand. Based on the analysis performed at the Regional Chemical Station in Wesoła, it was found that the soil was of neutral pH (pH in 1n KCL = 6.8), with a high content of humus (3.78%), available phosphorus($H_2PO_4^-$- 170 mg· kg^{-1}), magnesium (Mg^{2+} - 84 mg·kg^{-1}) and moderate content of total nitrogen (N – 1.3 g· kg^{-1}) and available potassium (K^+ - 114 mg· kg^{-1}). The following factors were examined:

- Type of biostimulant: Algex, Tytanit, Asahi SL and a control (no biostimulant addition);
- Nitrogen application rate: 0 (control) and 30 kg· ha^{-1}.

Algex is an extract of the sea alga *Ascophyllum nodosum* which contains vitamins, amino acids, phytohormones (auxins, cytokinins, gibberellins), polysaccharides, betaine as well as macro- and microelements such as N –8%, P – 3.6%, K –7%, B – 0.036%, Zn – 0.025%, Cu – 0.009%, Fe – 0.016%, Mn – 0.036% and Mo – 0.0036%. Algex is manufactured by Rosier, Moustier, Belgium.

Tytanit produced by INTERMAG Ltd., Olkusz, Poland. Tytanit contained 8.5 g Ti per 1 dm^3 (0.8% m/m), in the form of Ti-ascorbate.

Siedlce University of Natural Sciences and Humanities, 08-110 Siedlce, B. Prusa 14 st., Poland.

Corresponding Author: A. Godlewska, Siedlce University of Natural Sciences and Humanities, 08-110 Siedlce, B. Prusa 14 st., Poland. Email: godlewskaa@uph.edu.pl

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Asahi SL produced by Arysta Life Science Ltd., Warsaw, Poland. Asahi SL contained phenolic compounds: sodium para-nitrofenolate – 0.3%, sodium orto-nitrofenolate – 0.2%, sodium 5-nitroguaiacolate – 0.1%.

The sowing rate of *Trifoliumpratense* cv. Rozeta was calculated based on the standards drawn up by Institute for Land Reclamation and Grassland Farming (Jankowski *et al.*, 2005). Standard cultivation methods were applied in the experiment and, additionally, plants were fertilised with different biostimulants. The clover sowing rate was 21 kg·ha^{-1}. In 2013, when seeds were sown, neither biostimulants or nitrogen fertiliser was applied. In October 2013, only one cut was performed at the plant height of 10 cm. Over the study period (2014-2015), the cutting regime was three harvests per year. Ammonium nitrate was applied for every regrowth (three times per year). The total nitrogen amount was split into three equal rates, which were applied before each cutting. P and K fertilization was applied to all the plots. Phosphorus was applied once as triple superphosphate at a rate of 40 kg·ha^{-1}P_2O_5 in the spring. The amount of potassium (160 kg·ha^{-1}K_2O) was split into three equal rates and applied prior to each cutting as 60% potash salt. The biostimulants was sprayed as an aqueous solution (Algex - 1.90% solution, Tytanit - 0.15% solution, Asahi SL - 0.15% solution). The spraying was performed before each cutting: the first application was three weeks before the first cutting, the second one two weeks after the first harvest and the last one three weeks after the second harvest. Biostimulant rates following each cut were applied as recommended by the manufacturer that is thinned with water to obtain the volume of 400 dm^3: Algex - 6 dm^3·ha^{-1}, Tytanit – 0.4 dm^3·ha^{-1}, Asahi SL – 0.6 dm^3·ha^{-1}.

Leaf SPAD value was determined using a Minolta Chlorophyll Meter SPAD-502. It measures the light absorption by leaves at the wavelength of 650 and 940 nm. The quotients of light absorption at the two wavelengths are unitless and are called SPAD values. They reflect the leaf greenness index which corresponds to the total chlorophyll content (Pacewicz and Gregorczyk, 2009). Measurements were taken in each plot on 10 randomly selected alfalfa leaves (top leaves) the day before harvest.

During harvest of each cut, green matter from each plot (whole area, that is 10 m^2) was weighed to determine the yield and 0.5 kg green matter samples of alfalfa were taken to determine the drying-up coefficient and to carry out chemical analyses. The samples were left to dry in a ventilated room. Airy dry matter was weighed (to determine dry matter yield per plot) and was then shredded and ground. The obtained material was subjected to chemical analysis to determine dry matter (by determining moisture content), true protein and soluble carbohydrates. The method of determination was near-infrared spectroscopy (NIRS) using a NIRFlex N-500 spectrometer and ready-to-use INGOT calibration applications.

Statistical analysis was conducted using the program STATISTICA StatSoft, Inc. (2011). STATISTICA (data analysis software system), version 10 (www.statsoft.com). Significance of differences between means was checked using the Tukey test at the significance level of $\alpha \leq 0.05$.

Meteorological conditions during the study period were changeable and precipitation was very unevenly distributed (Table 1).

RESULTS AND DISCUSSION

Red clover dry matter (Table 2) differed significantly regardless of nitrogen regime and biostimulants applied. In the first study year, the yields were almost twice as high as the yields harvested in the second year, which was probably due to exceptionally low rainfall in April, June and August 2015 (Table 1).

Analysis of results averaged across two study years revealed significantly higher biomass yields of red clover (averaged 16.2%) fertilised with 30 kg N·ha^{-1} compared with yield performance in non-fertilised plots, regardless of the remaining factors.

Algex proved to be the best biostimulant as it significantly increased red clover biomass yields in each study year and at all nitrogen fertilisation levels, the increase

Table 1: Meteorological condition in years 2014-2015 by Meteorological station in Siedlce.

Years	Means monthly air temperatures (°C)						Means in growing season (IV-IX) (°C)
	Month						
	IV	V	VI	VII	VIII	IX	
2014	9.7	13.7	15.1	20.4	17.8	13.7	15.1
2015	8.1	12.3	16.5	14.3	21.1	8.8	13.5
Mean of many years (2006- 2015)	9.6	14.0	17.2	19.9	18.4	13.6	15.5
Years	Monthly precipitation (mm)						Sum in season (IV -IX) (mm)
	Month						
	IV	V	VI	VII	VIII	IX	
2014	39.5	79.3	50.3	62.5	66.3	26.7	324.6
2015	29.7	100.6	41.1	68.3	12.0	77.5	329.2
Mean of many years (2006- 2015)	26.9	68.9	64.6	55.8	65.3	44.3	325.8

Table 2: Dry matter yield of *Trifolium pretense* L. (t • ha^{-1}) (sum of three cuts).

Year	Dose of N kg•ha^{-1}	Without biostimulant (control)	Biostimulant			Mean
			Algex	Tytanit	Asahi SL	
2014	0	13.9 a	25.1 b	19.7 c	17.1 d	19.0 A
	30	16.3 a	26.7 b	23.8 c	17.1 d	21.0 A
2015	0	7.0 a	11.4 b	10.1 bc	9.1 abc	9.4 A
	30	10.0 a	15.1 b	11.8 a	11.1 a	12.0 B
2014	Mean	15.1 a	25.9 b	21.8 c	17.1 a	20.0 A
2015		8.5 a	13.3 b	11.0 c	10.1 ac	10.7 B
Mean	0	10.5 a	18.3 b	14.9 c	13.1 c	14.2 A
	30	13.2 a	20.9 b	17.8 c	14.1 a	16.5 B
Mean		11.8 a	19.6 b	16.4 c	13.6 a	15.3

Different lower-case letters within the same line indicate significant differences.

Different uppercase letters indicate that the values in the column for individual factors (Dose N and Year) and their interaction differ significantly.

Table 3: The effect of the biostimulants on dry matter yield of *Trifolium pratense* L. by cut and nitrogen fertilization (t • ha^{-1}) (mean of two years).

Cut	Dose of N kg•ha^{-1}	Without biostimulant (control)	Biostimulant			Mean
			Algex	Tytanit	Asahi SL	
1	0	2.1 a	3.6 b	2.8 ab	2.4 a	2.7 A
	30	2.6 a	4.9 b	3.3 a	3.1 a	3.5 B
2	0	4.8 a	8.8 b	7.5 c	7.4 c	7.1 A
	30	7.0 a	9.6 b	8.7 c	7.3 a	8.2 B
3	0	2.5 a	5.9 b	4.6 c	3.3 a	4.1 A
	30	3.5 a	6.4 b	5.9 b	3.7 a	4.9 B
1	Mean	2.4 a	4.3 b	3.1 a	2.8 a	3.1 A
2		5.9 a	9. 2 b	8.1 c	7.4 c	7.6 B
3		3.0 a	6.2 b	5.3 c	3.5 a	4.5 C
Mean	0	3.2 a	6.1 b	5.0 c	4.4 c	4.7 A
	30	4.4 a	7.0 b	6.0 c	4.7 a	5.5 B
Mean		3.8 a	6.5 b	5.5 c	4.5 a	5.1

Different lower-case letters within the same line indicate significant differences.

Different uppercase letters indicate that the values in the column for individual factors (Dose N and Cut) and their interaction differ significantly.

averaging 66.1% compared with control plots. Also reports by other authors pointed to a positive effect of sea algae extract on yield performance of crop plants (Kavitha *et al.*, 2008; Nerrisa *et al*., 2016; Godlewska and Ciepiela, 2016b). An application of Tytanit and Asahi SL was followed by a yield increase of 39.0 and 15.2%, respectively, compared with yields harvested in non-treated plots. Similar results were reported by Whitted-Haag (2014) and Przybysz *et al.* (2014). It should be noted that in units without nitrogen fertilisation but treated with biostimulants red clover yields were higher compared with non-treated plots fertilised with 30 kg N • ha^{-1}.

Red clover yields harvested with consecutive cuts (means across study years) differed significantly (Table 3). The lowest yields of the test plant were produced by clover harvested with the first cut and the highest with the second cut, the differences being statistically significant. Such wide differences may have been due to uneven distribution of atmospheric precipitation.

Statistical analysis demonstrated that nitrogen fertilisation significantly affected red clover biomass yields harvested with each cut, regardless of the remaining experimental factors.

The biostimulants contributed to an increase in red clover yield harvested with each cut although for Tytanit (the first cut) and Asahi SL (the first and third cut as well as the second cut harvested in plots without nitrogen fertiliser) the increase in biomass yield was insignificant.

The content of chlorophyll (Table 4) in red clover leaves was significantly affected by all the experimental factors. The highest and lowest average amounts of chlorophyll were recorded in the leaves of plants harvested with, respectively, the second and third cut, the differences being statistically significant.

The nitrogen rate of 30 kg•ha^{-1} contributed to a significant increase in the average chlorophyll content determined in red clover leaves. Only a slight increase in

the concentration of this pigment following nitrogen fertilisation was recorded in red clover leaves of the third cut and the differences were statistically insignificant. Also research by Peyvast *et al*. (2007) demonstrated that chlorophyll content in plant leaves increased rapidly following fertilisation.

Biostimulants Asahi SL had the most beneficial effect on chlorophyll concentration in plant leaves, the increase in the leaf content of chlorophyll averaging 7.99%. The results reported here agree with findings of other authors (Przybysz *et al*., 2014; Murawska *et al.*, 2017). The Biostimulants Algex and Tytanit increased chlorophyll content in red clover leaves by 3.63%, on average, compared with control, the differences being statistically insignificant. Similar relationships were described in works by Ambika and Sujatha (2017) and Kováčik et al. (2018).

The content of protein compounds in red clover dry matter was affected by all the experimental factors (Table 5). The content was the highest in plants harvested with the third cut regardless of nitrogen fertilisation and biostimulant application. This might have been due to poorer dry matter yield performance of red clover harvested with the third cut as well as weather conditions. Protein concentration in red clover increased at consecutive cuts. Similar findings being reported by Radkowski and Styrc (2009).

Analysis of the distribution of protein compounds across individual cuts demonstrated that nitrogen fertilisation significantly increased protein compounds in red clover only

Table 4: The effect of the biostimulants on value of SPAD in leaves of *Trifolium pratense* L. (value of SPAD) by cut and nitrogen fertilization (mean of two year).

Cut	Dose of N	Without biostimulant	Biostimulant			Mean
	kg·ha^{-1}	(control)	Algex	Tytanit	Asahi SL	
1	0	52.6 a	55.2 ab	55.1 ab	57.8 b	55.2 A
	30	55.1 a	57.4 ab	57.2 ab	59.8 b	57.4 B
2	0	54.0 a	56.5 ab	56.1 ab	57.9 b	56.1 A
	30	57.3 a	59.0 a	59.5 a	62.4 b	59.6 B
3	0	50.6 a	52.1 a	52.4 a	54.5 b	52.4 A
	30	52.9 a	54.4 ab	54.1 ab	56.2 b	54.4 A
Mean	0	52.4 a	54.6 b	54.5 b	56.7 c	54.6 A
	30	55.1 a	56.9 a	56.9 a	59.5 b	57.1 B
1	Mean	53.9 a	56.3 b	56.2 b	58.8 c	56.3 A
2		55.7 a	57.8 b	57.8 b	60.2 c	57.8 A
3		51.8 a	53.3 a	53.3 a	55.4 b	53.4 B
Mean		53.8 a	55.8 a	55.7 a	58.1 b	55.8

Different lower-case letters within the same line indicate significant differences.

Different uppercase letters indicate that the values in the column for individual factors (Dose N and Cut) and their interaction differ significantly.

Table 5: The effect of the biostimulants on content of true protein in *Trifolium pretense* L. by cut and nitrogen fertilization (g • kg^{-1}DM) (mean of two year).

Cut	Dose of N	Without biostimulant	Biostimulant			Mean
	kg·ha^{-1}	(control)	Algex	Tytanit	Asahi SL	
1	0	105.5 a	123.9 b	133.9 bc	135.0 c	124.6 A
	30	125.0 a	126.4 a	135.6 ab	142.6 b	132.4 A
2	0	116.1 a	122.0 a	135.9 b	145.8 b	130.0 A
	30	115.5 a	127.6 b	141.6 c	157.5 d	135.6 A
3	0	123.6 a	140.7 b	150.4 b	174.5 c	147.3 A
	30	141.7 a	159.4 b	169.3 b	183.1 c	163.4 B
Mean	0	115.1 a	128.9 b	140.1 c	151.8 d	133.9 A
	30	127.4 a	137.8 b	148.8 c	161.1 d	143.8 B
1	Mean	115.3 a	125.2 ab	134.8 b	138.8 bc	128.5 A
2		115.8 a	124.8 a	138.8 b	151.7 c	132.8 A
3		132.7 a	150.1 b	159.9 b	178.8 c	155.3 B
Mean		121.2 a	133.3 b	144.5 c	156.4 d	138.9

Different lower-case letters within the same line indicate significant differences.

Different uppercase letters indicate that the values in the column for individual factors (Dose N and Cut) and their interaction differ significantly.

Table 6: The effect of the biostimulants on content of soluble carbohydrates in *Trifolium pretense* L.by cut and nitrogen fertilization (g·kg^{-1}DM) (mean of two year).

Cut	Dose of N kg·ha^{-1}	Without biostimulant (control)	Biostimulant			Mean
			Algex	Tytanit	Asahi SL	
1	0	99.5 a	107.6 a	123.9 b	110.9 a	110.5 A
	30	84.5 a	95.7 ab	111.0 bc	100.5 b	97.9 B
2	0	70.9 a	76.4 ab	87.2 b	76.7 ab	77.8 A
	30	51.7 a	69.2 b	81.1 c	73.7 bc	68.9 B
3	0	74.8 a	75.4 a	98.7 b	85.2 a	83.5 A
	30	65.5 a	76.7 ab	97.1 c	86.6 abc	81.5 A
Mean	0	81.7 a	86.5 a	103.3 b	90.9 a	90.6 A
	30	67.2 a	80.5 ab	96.4 c	86.9 abc	82.8 B
1	Mean	92.0 a	101.7 b	117.5 c	105.7 b	104.2 A
2		61.3 a	72.8 ab	84.2 b	75.2 b	73.4 B
3		70.2 a	76.1 ab	97.9 c	85.9 b	82.5 C
Mean		74.5 a	83.5 ab	99.8 c	88.9 b	86.7

Different lower-case letters within the same line indicate significant differences.

Different uppercase letters indicate that the values in the column for individual factors (Dose N and Cut) and their interaction differ significantly.

in plants harvested with the third cut, the increase amounting to 10.3%.

The most beneficial concentration of protein in plants was obtained following an application of Asahi SL. The biostimulant contributed to an increase in the red clover content of protein compounds which averaged as much as 29.0% compared with non-treated red clover. Algex and Tytanit increased the concentration of protein in red clover by 9.98% and 19.2%, respectively. The authors whose works support the aforementioned findings include Pise and Sabale (2010), Haroun *et al*. (2011) and Hidangmayum and Sharma (2017).

The concentration of soluble carbohydrates in red clover (Table 6) was influenced by all the experimental factors. Soluble carbohydrates were significantly the highest in clover harvested with the first regrowth compared with the second and third cut which can be explained by the fact that, under conditions involving high temperatures (the second and third cut), plants utilise more soluble carbohydrates due to increased respiration which makes use of sugars. Similar findings were reported by Ciepiela and Godlewska (2019) in their research on Italian ryegrass.

Nitrogen fertilisation contributed to a decline in the average concentration of soluble carbohydrates in red clover, which amounted to 9.42%, compared with plants harvested in non-fertilised plots, the difference being statistically significant.

The biostimulant Tytanit contributed to a significant increase in the concentration of soluble carbohydrates in the test plants at each nitrogen fertilisation level and at all the cuts. The average increase was 34% compared with plots without biostimulant application. In plants treated with a sea algae extract, sugar content increased significantly only in second-cut red clover fertilised with nitrogen whereas Asahi SL displayed such an effect also for red clover harvested with the first cut in plots fertilised with nitrogen. Similarly, Zodape *et al*. (2010) and Haroun *et al*. (2011) confirmed a positive effect of sea algae extracts on the concentration of the analysed component in plants. In contrast, Cwalina-Ambroziak and Amarowicz (2012) reported the opposing results as, following Asahi SL application, they observed a decline in the amount of sugars in tomato and red pepper fruits.

CONCLUSION

Regardless of nitrogen fertilisation and biostimulant application, the amount of red clover yield and its quality differed significantly at individual cuts. Biostimulants significantly increased red clover biomass yields, the highest increase, amounting to 66.1%, being recorded following an application of Algex which is an extract of the sea algae *Ascophyllum nodosum*. Red clover yields were higher in plots treated with biostimulants but not fertilised with nitrogen compared with untreated units fertilised with nitrogen applied at the rate of 30 kg·ha^{-1}, which is the most beneficial in financial and ecological terms. All the examined biostimulants contributed to an increase in the amount of organic components in red clover dry matter. Nitrogen applied at the rate of 30 kg·ha^{-1} significantly increased red clover yielding, chlorophyll content in red clover leaves and protein compounds but it also significantly reduced soluble carbohydrates in the dry matter of test plants.

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