ASSESSMENT OF THE EFFECT OF VARIOUS BIOSTIMULANTS ON *MEDICAGO X VARIA* T. MARTYN YIELDING AND CONTENT OF SELECTED ORGANIC COMPONENTS

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Abstract. The objective of the present work was to determine the effect of various biostimulants on sand lucerne yielding and content of chlorophyll, proteins, and simple sugars against the background of nitrogen fertilisation regime. 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 Poland in the second decade of August 2013). 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}. Statistical analysis demonstrated that the biostimulants applied significantly increased plant biomass yields but the effect was more beneficial in plots to where no nitrogen had been applied. Chlorophyll content in lucerne leaves and monosaccharide content were significantly higher following an application of all the biostimulants. Their effect on the sand lucerne content of protein compounds varied. An application of Tytanit and Algex contributed to an increase in protein compounds in test plants but, for Algex, the differences were statistically insignificant. By contrast, an application of Asahi SL was followed by a significant decline in the concentration of protein compounds in lucerne plants. Significantly higher average dry matter yields, a higher average chlorophyll content and a higher average concentration of protein compounds were noted in plots fertilised with nitrogen compared with non-fertilised units. However, nitrogen fertilisation regime significantly reduced the sand lucerne content of simple sugars.
Keywords: *chlorophyll, protein compounds, monosaccharides, Algex, Tytanit, Asahi SL*

Introduction

At present, a challenge for scientists is to look for new technological solutions which would beneficially affect the growth, development, yielding and chemical composition of plants, coupled with limiting e.g. natural environment pollution, and production of healthy food. Recently, products which stimulate metabolic processes in plants, even if they are grown under unfavourable conditions, have been appearing, including biostimulants or growth stimulants (Kocira et al., 2016) of mineral or organic origin (alga and seaweed extracts, phytohormones, micro- and macroelements such as titanium). Biostimulants are products which contain biologically active substances which, by influencing metabolism, contribute to an increase in yields. As it is frequently accompanied by an improvement in yield quality (Kocira et al., 2015; Godlewska and Ciepiela, 2017), the products supplement an application of plant protection preparations and mineral fertilisation. When rationally used, they may be one of the main factors which positively affect the environment and soil productivity (Murawska et al., 2017). According to many authors, an application of biostimulants significantly contributes to an increase in yield, nutrient content, e.g. protein (Szabo et al., 2011), sugars (Godlewska and Ciepiela, 2016), and chlorophyll. It is well known that the effect of

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biostimulants depends on many factors, including crop plant species (Sultana et al., 2005). There are few reports in scientific literature on the effect of biostimulants in herbage legume cultivation, which prompted the authors to undertake research in this field.

The objective of the present work was to determine the effect of various biostimulants on sand lucerne yielding and content of chlorophyll, proteins, and simple sugars against the background of nitrogen fertilisation regime. Protein compounds and monosaccharides are basic components in an assessment of nutritional value of roughages. Chlorophyll content in lucerne leaves is indicative of photosynthesis which has a direct influence on plant growth and development.

Materials and methods

A field experiment was arranged as a randomised sub-block design (split-plot) with three replicates at the Siedlce Experimental Unit of the University of Natural Sciences and Humanities in Poland (52.169° N, 22.280° E) in mid-August 2013. The plot area was 10 m^2 (2.5 m × 4.0 m). According to the Polish soil classification system (Systematics of Polish Soils, 2011), the soil used in the experiment was Hortic Anthrosol (WRB), 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 reaction (pH in 1n KCL = 6.8), with a high humus content (3.78%), available phosphorus content ($H_2PO_4^-$ − 170 $mg{\cdot}kg^{-1}$) and magnesium content (Mg^{2+} − 84 $mg{\cdot}kg^{-1}$), and average total nitrogen (N – 1.3 $g{\cdot}kg^{-1}$) and available potassium (K^+ − 114 $mg{\cdot}kg^{-1}$) contents. 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 $kg{\cdot}ha^{-1}$

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

Asahi SL is produced by Arysta Life Science Ltd., Warsaw, Poland. The product contains the following phenolic compounds: sodium p-nitrophenolate − 0.3%, sodium o-nitrophenolate − 0.2% and sodium 5-nitroguaiacolate − 0.1%.

The sowing rate of *Medicago x varia* T. Martyn cv. Marshal was calculated based on the standards drawn up by IMUZ (the 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 lucerne sowing rate was 23 $kg{\cdot}ha^{-1}$. 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 (2014-2016), the cutting regime consisted of three harvests per year. Ammonium nitrate was applied three times per year. The total nitrogen amount was split into three equal rates which were applied before each cutting. P and K fertilisation was applied to all the plots. Phosphorus was

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applied once as triple superphosphate at the 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. 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 chlorophyll 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 (Machul, 2005; Pacewicz and Gregorczyk, 2009). Measurements were taken in each plot on 10 randomly selected sand lucerne 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 lucerne 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), protein compounds, and monosaccharides. 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 by means of the program STATISTICA StatSoft, Inc. (2011). STATISTICA (data analysis software system), version 10 (www.statsoft.com) was used. 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*). The mean air temperature in the 2015 growing season was by 2 degrees lower than the mean across 2006-2016 whereas precipitation sum in 2016 was by 24 mm higher than the value for the preceding decade. July 2016 was of particular interest as the precipitation sum was twice as high as the mean across 2006-2016 and amounted to 25.6% of the precipitation sum for the whole growing season. By contract, unusual precipitation shortages occurred in September 2014, April and July 2015, and May and September 2016.

Table 1. *Meteorological condition in years 2014-2016 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
2016	8.8	14.6	18.1	19.0	17.9	14.4	15.5
Mean of 2006-2016	9.6	14	17.2	19.9	18.4	13.6	15.5

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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	77.5	329.2
2016	55.1	36.8	56.9	112.5	73.3	15.2	349.8
Mean of 2006-2016	26.9	68.9	64.6	55.8	65.3	44.3	325.8

Results and discussion

An analysis of average results for three study years demonstrated significant variation in sand lucerne yields regardless of the experimental factors (*Table 2*).

Table 2. *Dry matter yield of Medicago x varia T. Martyn (t ha^{-1}) (sum of three cuts)*

Year	Dose of N kg ha^{-1}	Biostimulant				Mean
		Without biostimulant (control)	Algex	Tytanit	Asahi SL	
2014	0	9.6 a	14.7 b	13.1 bc	12.7 c	12.5 A
	30	15.3 a	15.7 a	16.1 a	20.3 b	16.9 B
2015	0	18.2 a	20.5 b	20.1 b	18.7 ab	19.4 A
	30	21.7 a	23.1 a	23.3 a	22.9 a	22.8 B
2016	0	12.7 a	18.8 b	16.8 c	14.3 a	15.7 A
	30	19.5 a	20.6 ab	21.5 b	24.0 c	21.4 B
2014	Mean	12.5 a	15.2 bc	14.6 b	16.5 c	14.7 A
2015		20.0 a	21.8 a	21.7 a	20.8 a	21.1 B
2016		16.1 a	19.7 b	19.2 b	19.1 b	18.5 C
Mean	0	14.0 a	18.7 b	17.3 b	15.5 a	16.4 A
	30	19.5 a	20.6 a	21.2 ab	23.0 b	21.1 B
Mean		16.8 a	19.7 b	19.3 b	19.3 b	18.9

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 Years) and their interaction differ significantly

Significantly the lowest biomass yields were obtained in the first study year, them being the highest in the second year. Precipitation amount is an important factor which determines yield levels. Thus, it can be inferred that such low yields in the first study year may have been due to relatively the lowest precipitation amounts (*Table 1*). An application of biostimulants, regardless of the study years and nitrogen fertilisation regime, increased plant yields (by 15.6%, on average). The effect of biostimulants in the individual study years and in combination with nitrogen rates was equivocal. In non-fertilised plots, the biostimulants Algex and Tytanit contributed to a significant increase in lucerne yields in each study year compared with yields harvested from plots not treated with the biostimulants. Such an effect for Asahi SL was observed in the first study year only. A significant rise in yields obtained from plots fertilised with nitrogen

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was observed following an application of Asahi SL (in the first and third study year) and Tytanit (in the third year). Also Tan et al. (2011), Kováčik et al. (2018) and Whitted-Haag et al. (2014) obtained significant plant yield increases following an application of Tytanit. In each study year, the biostimulant Algex increased lucerne yields in plots fertilised with nitrogen but the differences were insignificant, which is in disagreement with results obtained by other researchers. Many authors have reported a significant effect of an application of sea alga extract on plant yields (Kumar and Sahoo, 2011; Sabir et al., 2014; Nerrisa et al., 2016). However, one should bear in mind that the effect of natural biostimulants is to a great extent determined by crop plant species and cultivar (Sultana et al., 2005; Godlewska and Ciepiela, 2016). Regardless of the remaining factors, nitrogen fertilisation regime significantly influenced yields produced by the test plant.

Dry matter yield (t ha^{-1}) harvested with individual cuts as affected by nitrogen fertilisation regime and type of biostimulant is presented in *Table 3*.

Table 3. *Dry matter yield of Medicago x varia T. Martyn (t ha^{-1}) (average of three years)*

Cut	Dose of N kg ha^{-1}	Biostimulant				Mean
		Without biostimulant (control)	Algex	Tytanit	Asahi SL	
1	0	3.3 a	5.0 b	4.1 c	3.4 a	4.0 A
	30	5.1 a	5.5 a	5.6 a	5.4 a	5.4 B
2	0	6.5 a	8.6 b	8.4 b	7.6 c	7.8 A
	30	9.1 a	9.3 a	9.7 a	11.5 b	9.9 B
3	0	4.3 a	5.1 b	4.7 ab	4.5 ab	4.7 A
	30	5.3 a	5.8 ab	5.9 ab	6.1 b	5.8 B
1	Mean	4.2 a	5.2 b	4.9 bc	4.4 ac	4.7 A
2		7.8 a	9.0 b	9.1 b	9.5 b	8.8 B
3		4.8 a	5.4 a	5.3 a	5.3 a	5.2 A
Mean	0	4.7 a	6.2 b	5.8 bc	5.2 ac	5.5 A
	30	6.5 a	6.9 a	7.1 ab	7.7 b	7.0 B
Mean		5.6 a	6.6 b	6.4 b	6.4 b	6.3

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 Cut) and their interaction differ significantly

Regardless of the experimental factors, the lowest lucerne yields were harvested in the first cut, them being the highest for the second cut (mean across years), which should be linked with the meteorological conditions (*Table 1*). Irrespective of the biostimulants, nitrogen regime increased yields harvested in each cut. The effect of the biostimulants on lucerne yields was favourable but the differences were not always significant. Regardless of the N fertiliser factor, in the first and second cut, the biomass of plants in plots treated with biostimulants was significantly higher compared with control yields. In individual cuts, the effect of biostimulants as influenced by nitrogen regime was equivocal. In units without nitrogen application, a significant increase in yield was observed for the first cut following an application of Algex and Tytanit, for the second

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cut following spraying with all the biostimulants, and for the third cut after Asahi SL had been applied. Significantly higher yields harvested in plots fertilised with nitrogen were recorded for the second and third cut only after spraying with Asahi SL compared with control yields.

The nitrogen status of plants, important in terms of the effectiveness of crop plant production and yield quality, can be determined by means of an application of a test which measures the chlorophyll content in leaves (Rostami et al., 2008). Chlorophyll content in the leaves of sand lucerne harvested in individual cuts and study years as affected by nitrogen fertilisation and biostimulant application is presented in *Table 4*. Data analysis revealed that nitrogen regime increased SPAD in lucerne leaves by 7.66%, the differences being significant. In their study, Szulc et al. (2012) found similar relationships. The effect of biostimulants examined in the experiment reported here was unidirectional. Regardless of the nitrogen regime, cuts and study years, each biostimulant significantly increased chlorophyll content in test plants (by 6.70%, on average). The obtained results correspond to data presented by other authors. Murawska et al. (2017) applied Asahi SL, a sea alga extract and Tytanit, and observed a clear increase in chlorophyll content in winter wheat leaves. Also, El-Miniawy et al. (2014) demonstrated in their work a banaficial effect of a sea alga extract on SPAD in strawberry leaves. Research by other workers revealed that an application of Tytanit increased chlorophyll content in the leaves of timothy (Radkowski, 2013), winter wheat and winter oilseed rape (Kováčik etal., 2014).

Table 4. *The effect of the biostimulants on content of chlorophyll in leaves of Medicago x varia T. Martyn (value of SPAD) by nitrogen fertilization, cut and study year*

Tested trait		Biostimulant				Mean
		Without biostimulant (control)	Algex	Tytanit	Asahi SL	
Dose of N kg ha^{-1}	0	51.7 a	56.3 b	55.4 b	55.8 b	54.8 A
	30	56.7 a	58.7 b	59.7 bc	61.0 c	59.0 B
Cut	1	53.7 a	56.3 b	56.3 b	57.6 c	56.0 A
	2	57.5 a	59.8 b	60.6 b	59.9 b	59.4 B
	3	51.5 a	56.5 b	55.8 b	57.9 c	55.4 A
Year	2014	54.3 a	55.8 b	56.8 b	56.8 b	55.9 A
	2015	54.9 a	57.9 b	57.7 b	57.8 b	57.1 B
	2016	53.7 a	57.9 b	57.8 b	59.4 c	57.2 B
Mean		54.2 a	57.4 b	57.6 b	58.5 b	56.9

Different letters within the same line indicate significant differences. Values in column for individual factors indicated with different, capital letters differ significantly

Chlorophyll content in the plants of individual cuts (mean across years) changed during the growing period, it being significantly the highest in the second cut lucerne. Also, analysis of the obtained results demonstrated that, in each cut, an application of biostimulants significantly increased chlorophyll content in lucerne compared with control plants which had not been treated with biostimulants. The biostimulant Asahi SL significantly increased chlorophyll content in the first and third cut lucerne leaves compared with all the remaining treatments. Moreover, the chlorophyll content in

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lucerne leaves was observed to increase in individual study years. Similar relationships were reported in the works by Olszewska (2008) and Ciepiela et al. (2013). Soil moisture may be one of the factors affecting chlorophyll content in plants. Thus, the highest chlorophyll content in the second cut plants may have been due to a large amount of precipitation accompanied by high 24-hour air temperatures during the period of growth of these plants.

The sand lucerne content of protein compounds in dry matter averaged 176.3 $g{\cdot}kg^{-1}$ DM (*Table 5*) and was affected by all the experimental factors. Regardless of biostimulant application and nitrogen regime, significantly the lowest level of protein compounds was recorded for the second cut lucerne, it being the highest for the third cut plants. When study years are taken into consideration, the lowest concentration of protein compounds was determined in the second cut plants, it being the highest in the third cut.

Table 5. *The effect of the biostimulants on content of protein compounds in Medicago x varia T. Martyn by nitrogen fertilization, cut and study year (g kg^{-1} DM)*

Tested trait		Biostimulant				Mean
		Without biostimulant (control)	**Algex**	**Tytanit**	**Asahi SL**	
Dose of N kg ha^{-1}	0	165.7 a	172.3 ab	177.2 b	154.8 c	167.5 A
	30	185.4 a	191.9 ab	196.8 b	181.9 ac	189.0 B
Cut	1	174.2 a	180.3 abc	186.7 b	171.6 ac	178.2 A
	2	156.1 a	163.3 ab	168.3 b	150.8 ac	159.6 B
	3	196.3 a	202.6 ab	206.1 b	182.6 c	196.9 C
Year	2014	165.2 a	169.9 ab	176.2 b	155.6 ac	166.7 A
	2015	152.2 a	159.3 ab	164.7 b	143.9 ac	155.0 B
	2016	194.5 a	201.3 ab	205.5 b	188.8 ac	197.5 C
Mean		175.5 a	182.1 ab	187.0 b	168.3 c	176.3

Different letters within the same line indicate significant differences. Values in column for individual factors indicated with different, capital letters differ significantly

The effect of biostimulants varied considerably. The most beneficial effect of Titanit was observed on protein compound content in sand lucerne. The biostimulant significantly increased (by 6.55%, on average) this content compared with control plants. The results correspond to findings reported by Murawska et al. (2017) who observed an increase in the winter wheat grain content of protein after spraying with Tytanit. An application of Algex contributed to an increase in protein compounds compared with their concentration in control plants (by 3.76%, on average) but the differences were statistically insignificant. However, a significant effect of sea alga extracts has been reported in many works (Matysiak et al., 2012; Ciepiela and Godlewska, 2014). It may follow from the fact that components in sea alga extracts (auxins, cytokinins, gibberellic acid and amino acids) increase plant physiological activity including protein synthesis (Szabo et al., 2011).

The biostimulant Asahi SL applied in the experiment contributed to a decline in protein compound content (by 4.28%, on average) in sand lucerne plants harvested in each cut (mean across years) compared with control plants. Significant differences

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between values reflecting the decline in the content of this component were recorded for plants harvested in the third cut only.

Regardless of biostimulant application, cuts and study years, nitrogen fertilisation regime significantly increased the concentration of protein compounds in lucerne (by 12.8%, on average) compared with the content in lucerne harvested in plots where no nitrogen had been applied.

Monosaccharide content (*Table 6*) was significantly the highest in the dry matter of the first cut sand lucerne (mean across years) compared with plants in the second and third cut. This was due to the fact that these compounds were used in large amounts during increased plant respiration under the conditions characterised by high temperatures (the second and third cut) when sugars are utilised. The findings correspond to results of the study reported by Ciepiela and Godlewska (2014). Moreover, statistical analysis confirmed significantly the highest concentration of simple sugars in plants harvested in the third study year compared with the first and second study year.

Table 6. *The effect of the biostimulants on content of monosaccharides in Medicago x varia T. Martyn by nitrogen fertilization, cut and study year (g kg^{-1} DM)*

Tested trait		**Biostimulant**				**Mean**
		Without biostimulant (control)	**Algex**	**Tytanit**	**Asahi SL**	
Dose of N kg ha^{-1}	0	51.9 a	58.3 b	62.4 bc	55.0 ab	56.9 A
	30	38.8 a	45.8 b	49.5 bc	43.6 ab	44.4 B
Cut	1	62.5 a	70.2 b	74.7 bc	67.3 ab	68.7 A
	2	34.3 a	39.8 b	42.7 bc	36.7 ab	38.4 B
	3	39.3 a	46.3 b	50.4 bc	44.0 ab	45.0 C
Year	2014	39.5 a	47.0 ab	54.7 b	45.1 ab	46.5 A
	2015	42.6 a	49.4 ab	53.7 b	47.1 ab	48.2 A
	2016	49.2 a	55.6 b	57.9 b	52.2 ab	53.7 B
Mean		45.4 a	52.1 b	56.0 b	49.3 c	50.2

Different letters within the same line indicate significant differences. Values in column for individual factors indicated with different, capital letters differ significantly

Biostimulants tested in this study significantly increased monosaccharide content in lucerne compared with control plants (by 10.6%, on average), regardless of nitrogen application regime, cuts and study years. An application of Algex and Tytanit to plots which were and were not fertilised with nitrogen contributed to a significant increase in the lucerne content of monosaccharides. A beneficial effect of sea alga extracts on the concentration of simple sugars in plants has been confirmed in research by other authors (Roussos et al., 2009; Pacholczak et al., 2012; Godlewska and Ciepiela, 2013; El-Miniawy et al., 2014). However, there is no data available for sand lucerne. The biostimulant Asahi SL increased monosaccharide content in plants, too, but the differences were insignificant. Contrasting findings were reported by Cwalina-Ambroziak and Amarowicz (2012) who applied Asahi SL and found a decline in sugar content in tomato and red pepper fruit.

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Regardless of biostimulants, nitrogen fertilisation regime significantly reduced monosaccharide content in lucerne dry matter, which corresponds to research results reported by Godlewska and Ciepiela (2013).

Conclusions

Statistical analysis demonstrated that the biostimulants applied significantly increased plant biomass yields but the effect was more beneficial in plots to where no nitrogen had been applied. Chlorophyll content in lucerne leaves and monosaccharide content were significantly higher following an application of all the biostimulants. Their effect on the sand lucerne content of protein compounds varied. An application of Tytanit and Algex contributed to an increase in protein compounds in test plants but, for Algex, the differences were statistically insignificant. By contrast, an application of Asahi SL was followed by a significant decline in the concentration of protein compounds in lucerne plants. Significantly higher average dry matter yields, a higher average chlorophyll content and a higher average concentration of protein compounds were noted in plots fertilised with nitrogen compared with non-fertilised units. However, nitrogen fertilisation regime significantly reduced the sand lucerne content of simple sugars.

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