Scientific Papers. Series A. Agronomy, Vol. LXIII, No. 1, 2020
ISSN 2285-5785; ISSN CD-ROM 2285-5793; ISSN Online 2285-5807; ISSN-L 2285-5785

INFLUENCE OF NEW BIOSTIMULANTS ON AMINO ACID SYNTHESIS IN ALFALFA UNDER CLIMATE CHANGING CONDITIONS

Atanas SEVOV, Adelina HARIZANOVA, Georgi STANCHEV, Lyubka KOLEVA-VALKOVA

Agricultural University of Plovdiv, 12 Mendeleev Blvd, Plovdiv 4000, Bulgaria

Corresponding author email: asevov@yahoo.com

Abstract

Discussion on the impact of climate change on various aspects of life is of increasing concern to the scientific community. Providing food for the growing population in adverse climatic conditions is a major challenge. This includes the provision of high-quality animal feed. Applying environmental approaches in this direction is key to restoring ecological balance. In this study, we investigated the effect of novel biostimulants on the synthesis of amino acids in alfalfa. Green mass and alfalfa hay are high in protein and essential amino acids, the main indicators that determine the quality of the biomass produced. The use of biostimulants in this study leads to an improvement in the biological value of proteins - the total amount of essential amino acids is increased and the ratio of essential amino acids to the other proteinogenic amino acids is changed in favor of the essential and increased the nutritional value of feed. The results obtained are crucial for the successful implementation of green practices in agriculture.

Key words*: alfalfa, amino acid content, biostimulants, climate change, green agriculture.*

INTRODUCTION

Current methods in the development of alfalfa as a crop are directed at the use of mechanisms for direct impact on production and quality. In the cultivation of various high quality alfalfa varieties, techniques are used to directly influence the protein content, fiber (Petkova et al, 2018; Toktarbekova et al., 2020) or by increasing the percentage of leaves (Kephart et al., 1990; Huset et al., 1991). Modern varieties and hybrids have high productive potential, which is not fully realized in production conditions. The factors limiting the expression of their productive capacities are various, but among the most important are the environmental conditions and the applied agricultural technology. The use of modern techniques to improve the adaptability of plants to changing environmental conditions is an important prerequisite for nature conservation in the development of sustainable agriculture. Different products are used, which are a variety of chemicals applied to plants or soils to improve crop viability, yield, quality and resistance to abiotic stress. Biostimulants influence by improving plant metabolism leading to increased yields; increasing the resistance of plants to abiotic factors (meteorological factors, pollution, pesticides, etc.); facilitate the assimilation of trace elements and digestibility of nutrients; increase the quality of production and more, (Niewiadomska et al., 2019; 2020). Feeding plants is a specific process, the primary place where nitrogen occupies. In legumes, the basic amount of the element is ensured by biological fixation of atmospheric nitrogen. The advantage of legumes is that they can grow on soils with a very low concentration of bound nitrogen, where other plants cannot. By increasing mineral nitrogen in the soil (ammonium and nitrate), its absorption from nitrogen-fixing plants increases and symbiotic nitrogen fixation decreases (Blumenthal et al., 1999; Campbell, 1999; Ruselle and Birr, 2004). In addition to environmental factors, productive potential is also influenced by the age of the alfalfa crop and the intensity of use (Brink et al., 2010).

Two different enzymatic systems may be involved in the uptake of nitrogen by legumes. One is the nitrogenase system, which is part of the arsenal of symbiotic microorganisms and provides the fixation of atmospheric nitrogen (Ruselle and Birr, 2004; Hristozkova et al., 2010; 2011). The second system involves

nitrate reductase, which is responsible for the reduction of nitrate nitrogen from soil (Ruselle and Birr, 2004; Hristozkova et al., 2011; Petkova et al., 2018; Nedyalkova et al., 2019). In the subsequent steps for the primary incorporation of the resulting ammonium ions into an organic molecule, the enzymes glutamine synthetase and asparagine synthetase take part (Hristozkova et al., 2011). The end result is a large number of amino acids and amides, and their quality and quantity depend on environmental factors and the physiological state of the plants. Knowledge of the processes involved in their production enables them to be modulated and to improve the quality of the final output. For example, in addition to molybdenum, induction of nitrate reductase activity is induced by cytokinins, a combination of cytokinins and gibberellins, humic acids, carbohydrates, mainly glucose and sucrose, certain organic acids, ammonium sulfate, organic acids included in the Krebs cycle (Aranjielo et al., 2011).

The possibility of regulating the biosynthesis of amino acids and proteins in legumes through the use of biostimulants and leaf fertilizers (containing microelements, phytohormones and other growth regulators) in the face of changing climatic factors is a challenge in modern agriculture. This determined the purpose of this study on the effects of new biostimulants on the quantity and quality of amino acids in alfalfa.

During 2017-2019 period at the experimental field of the Agricultural University of Plovdiv it was conducted a field experiment under the following conditions: alluvial-meadow soil type of pH - 6.7-7.1 (H_2O) and medium level of basic nutritive elements.

The investigation was performed by the 4 replications block method in 10 m^2 lots.

The sowing was done in spring 2017 and the plants are treated by generally accepted technology for alfalfa forage production (Yankov et al., 1996).

During the investigation 4 variants were controlled: 1. treated by Tecamin max; 2. treated by Amino Bore; 3. treated by Plantafol; 4. treated by Fertigrain Foliar.

Tecamin max contains amino acids 14.4% of which free 12%, organic matter 60%, total nitrogen 7%. It activates the growth and development of crops, promotes the restoration of plants after stressful situations – frost, hail, herbicide effect, phytotoxicity, and helps for the transport in plants of mineral nutrients, including trace elements. Also, it increases plant productivity and yield and improves product quality.

AminoBore is an organic biostimulant containning nitrogen 4%, water soluble boron (B) 9% and free amino acids 5%. It is used in oilseeds, alfalfa, fruits, vineyards and more (Meets organic farming standard NFU 42-003-2). It promotes faster absorption and movement of boron in the plant, restoration of cultures under stress of abiotic character (cold, drought, hail) stimulation of photosynthesis and fruit formation, overall balanced development of crops.

Plantafol is a N:P:K 20:20:20 mineral leaf fertilizer enriched with micro elements chelated with an EDTA chelating agent. Its content includes total nitrogen 20% (of which nitrate nitrogen - 4%, ammonium nitrogen - 2% and amide nitrogen - 14%), phosphorus (as diphosphorus pentoxide) - 20%, potassium (as potassium sulfate) - 20%. Of the trace elements, boron (B) - 0.02%, copper (Cu) - 0.05%, iron (Fe) - 0.1%, manganese (Mn) - 0.05% and zinc (Zn) - 0.05% are present.

Fertigrain Foliar - biostimulator for leaf application. It contains amino acids 10%, nitrogen 5%, organic matter 40%, zinc 0.75%, manganese 0.50%, boron 0.10%, iron 0.10%, copper 0.10%, molybdenum 0.02% and cobalt 0.01%. It has a powerful effect of stimulating plant growth and development of the plants due to the unique combination of organic nutrients in the form. Free amino acids and the most important trace elements in the form of chelates are the starting components for protein and enzyme biosynthesis.

The treatment of each swath was performed at stage by 2 l/ha of the preparations.

MATERIALS AND METHODS

Plant material

Mnogolistna 1. The variety is representative of the newest generation of multifaceted alfalfa. Over 50% of the leaves of the plants hold from 5 to 7 petals on a single leaf handle.

Legend. The variety is registered by the US company Land O'Lakes. It is part of the new generation of so-called multifaceted alfalfa

with more than 3 leaf handles, and has better in vitro digestibility than standard three-leaf sorts.

Used preparations

Four leaf treatments were used, with different contents and a combination of active substances at a dose of 3 l/ha twice. The products were Tecamine Max, Amino Boron, Plantafol and Fertigrain Foliar.

Samples for enzymatic analyzes (roots with nodules and aboveground part) were collected in the budding and flowering phases when nitrogen fixation was most intense. The activity of four key enzymes of nitrogen assimilation - nitrogenase, glutamine synthetase, asparagine synthetase and nitrate reductase - was investigated.

Nitrogenase activity (EC 1.7.99.2.) was determined by the method of Hardy et al. (1973) with modification (Popov et al., 1985).

The activity of glutamine synthetase (EC 6.3.1.2.) was determined by orthophosphate separated from ATP, which was determined by the Sumner method (Evstigneeva et al., 1980).

Asparagin synthetase activity (EC 6.3.5.4.) was determined by the same procedure as for the determination of the enzyme glutamine synthetase, except that glutamate is replaced by aspartate in the incubation mixture.

Nitrate reductase activity. Nitrate reductase (EC 1.6.6.2.) catalyzes the reduction of nitrates to nitrites. The method for determining the amount of nitrite is based on the color complex formed by the interaction of the nitrite ions with sulfanylamide in acetic acid and with N-(1-naphthyl)-ethylenediamine (Berova et al., 2013).

Samples for analysis of the type and amount of amino acids (roots with tubers and aboveground part) were collected in the budding and flowering phases, when nitrogen fixation was most intense. The research was done in an accredited laboratory at the Agricultural University of Plovdiv. Measurement of amino acids was done with an automatic amino analyzer.

Statistical processing

The obtained data were mathematically processed by the method of variance analysis using the SPSS program, and the Dunkan multivariate test with the smallest significant difference (LSD) - 0.05 (5%) was used to determine the differences between the tested variants. Correlation analysis was performed with the SPSS program.

RESULTS AND DISCUSSIONS

The production, quality and longevity of alfalfa depend on both external (environmental conditions) and internal (genetically determined) factors. These factors are in complex relationships and the elimination of any of them reduces the effect of the others and ultimately affects both the yield and the longevity of the alfalfa crops.

Research on alfalfa shows that yields have increased by 20% over the last hundred years (Kertikova, 2000), with only 10% of this increase being due to genetic improvements. As a protein culture, in addition to yield, protein content, and in particular the amino acid composition, is also essential.

Alfalfa green mass and hay are characterized by high protein content and essential amino acids, essential indicators that determine the quality of the produced biomass.

Table 1. Essential amino acids content in Mnogolista 1 biomass (% by weight of dry matter) average for the study period

Variants Amino acids	Control	Tecamin Max	Amino Bore	Plantafol	Fertigrein Foliar
Lysine	1.71^{b}	1.72^{b}	1.56^{c}	1.57^{c}	1.92^{a}
Threonine	1.12^{b}	1.14^{b}	1.13^{b}	1.14^{b}	1.25^{a}
Valine	1.12^{c}	1.26^{b}	1.19^{c}	1.23^{b}	1.35^{a}
Methionine	0.13^{a}	0.11^{b}	0.09^{c}	0.09^{c}	0.11^{b}
Isoleucine	0.84^{c}	0.98^{ab}	0.89^{bc}	0.91^{b}	1.05^{a}
Leucine	1.71^{b}	1.78^{b}	1.57^{c}	1.57^{c}	1.86^{a}
Phenylalanine	1.22^{b}	1.24^{b}	1.19^{c}	1.21^{bc}	1.45^{a}
Total	7.85^{c}	8.23^{b}	7.62^{c}	7.72^{c}	8.99^{a}

The different letters (a, b, c) after the average show statistically significant differences between the analyzed variants.

A number of scientific studies show that the application of different growth regulators has a positive effect on the quality of alfalfa forage (Wang et al., 2003; Radu et al., 2010).

Proteinogenic amino acids in the alfalfa vegetative mass are related to the biological value and protein balance. Their amount in the individual protein fractions is under genetic control and difficult to change, but the ratio of protein fractions, and through them, the quality of the protein can change under external influences. The application of the tested products in the present study positively affects the total content of proteinogenic amino acids in both varieties. At Mnogolistna 1 variety, the application of different products significantly increased by 14.52% the content of essential amino acids in only one variant (Fertigrein Foliar), in Tecamin Max the increase was insignificant, and in the variants Amino Bore and Plantafol it was lower than the control (Table 1).

The results are similar for the other proteinogenic amino acids, except for the Plantafol variant wich has a 3.3% higher total amino acid composition, obtained mainly because of the aspartic acid. All tested amino acids, show higher content when Fertigrein Foliar is applied - aspartic acid by 27%, glycine by 21.8%, arginine by 21.4%, valine by 20.5% compared to the control (Table 2).

The reaction to the used products shows also variety differences. In the second alfalfa variety - Legend, a higher effect of treatment was observed with the Tecamin Max variant both for total proteinogenic and irreplaceable proteinogenic amino acids (Table 3).

Table 2. Total proteinogenic amino acids content in the alfalfa biomass at Mnogolistna 1 (% by weight of dry matter), averaged for the study period

Variants / Amino acids	Control	Tecamin Max	Amino Bore	Plantafol	Fertigrein Foliar
Lysine	1.71^{b}	1.72^{b}	1.56^{c}	1.57^{c}	1.92^{a}
Histidine	0.57^{b}	0.61^{a}	0.59^{ab}	0.59^{ab}	0.64^{a}
Arginine	1.31^{c}	1.49^{b}	1.11^{e}	1.25^{d}	1.59^{a}
Aspartic acid	3.11^{c}	3.29^{c}	3.34^{bc}	4.24^{a}	3.95^{b}
Threonine	1.12^{b}	1.14^{b}	1.13^{b}	1.14^{b}	1.25^{a}
Serine	1.33^{b}	1.31^{b}	1.44^{a}	1.47^{a}	1.45^{a}
Glutamic acid	2.49^{b}	2.61^{ab}	2.29^{c}	2.36^{b}	2.84^{a}
Proline	1.99^{a}	1.97^{a}	1.99^{a}	1.83^{b}	1.83^{b}
Glycine	1.01^{b}	1.17^{a}	0.98^{b}	1.04^{ab}	1.23^{a}
Alanine	1.29^{b}	1.41^{a}	1.27^{b}	1.23^{b}	1.52^{a}
Cysteine	0.09^{a}	0.09^{a}	0.11^{a}	0.08^{a}	0.09^{a}
Valine	1.12^{c}	1.26^{b}	1.19^{c}	1.23^{b}	1.35^{a}
Methionine	0.13^{a}	0.11^{b}	0.09^{c}	0.09c	0.11^{b}
Isoleucine	0.84^{c}	0.98^{ab}	0.89^{bc}	0.91^{b}	1.05^{a}
Leucine	1.7^{b}	1.78^{b}	1.57^{c}	1.57^{c}	1.86^{a}
Tyrosine	0.76^{b}	0.81^{a}	0.71^{b}	0.71^{b}	0.86^{a}
Phenylalanine	1.22^{b}	1.24^{b}	1.19^{c}	1.21^{bc}	1.45^{a}
Total	21.79^{c}	22.99^{b}	21.45^{c}	22.52^{b}	24.99^{a}

The different letters (a, b, c) after the average show statistically significant differences between the analyzed variants.

Table 3. Average content of essential and nonessential amino acids in aboveground biomass of alfalfa Legend variety (% by weight of dry matter)

Variants / Amino acids	Control	TecaminMax	AminoBore	Plantafol	FertigreinFoliar
Lysine	1.52^{c}	1.86^{a}	1.66^{b}	1.61^{b}	1.66^{b}
Threonine	0.97^{b}	1.23^{a}	1.12^{a}	1.07^{b}	1.06^{b}
Valine	1.09^{c}	1.46^{a}	1.27^{b}	1.32^{b}	1.22^{bc}
Methionine	0.04^{b}	0.09^{a}	0.06^{ab}	0.07^{a}	0.03^{b}
Isoleucine	0.88^{c}	1.11^{a}	0.91^{b}	1.06^{a}	0.92^{b}
Leucine	1.51^{bc}	1.91^{a}	1.56^{b}	1.8^{a}	1.61^{b}
Phenylalanine	1.11^{b}	1.31^{a}	1.26^{a}	1.24^{ab}	1.16^{b}
Total	7.12^{b}	8.97^{a}	7.84^{b}	8.17^{a}	7.66^{b}

The different letters (a, b, c) after the average show statistically significant differences between the analyzed variants.

The total irreplaceable amino acids content increases from 7.58% at Fertigrein Foliar up to 26% at Tecamin Max. The valine content increased the most - by 34% and the lowest of phenylalanine - by 18% (Table 3).

For other proteinogenic amino acids, the increase was between 8.09% for Fertigrein Foliar and 24.54% for Tecamin Max. The content of aspartic acid was significantly increased (by 77%) in the Tecamin Max treated variant, 57.8% in the Amino Bore variant, 31.0% in the Plantafol variant, 46.3% in the Fertigrein Foliar variant (Table 4).

At Legend variety, from all tested 17 proteinogenic amino acids, only proline reduced its content, and it significantly decreased from 25.2 to 28.73%. The most significant decrease was observed when Plantafol was applied (Table 4). Proline is thought to be a stress indicator amino acid and its amount increases with different types of stress. For water stress, for example, the higher the content of endogenous proline is in bean leaves, the greater is the decrease in water potential (Zlatev, 2005).

Table 4. Total proteinogenic amino acids content in the biomass of alfalfa Legend variety (% by weight of dry matter), average for the study period

Variants / Amino acids	Control	Tecamin Max	AminoBore	Plantafol	Fertigrein Foliar
Lysine	1.52^{c}	1.86^{a}	1.66^{b}	1.61^{b}	1.66^{b}
Histidine	0.54^{b}	0.66^{a}	0.66^{a}	0.57^{b}	0.58^{ab}
Arginine	1.21^{c}	1.47^{a}	1.29^{c}	1.29^{c}	1.31^{b}
Aspartic acid	2.42^{c}	4.3^{a}	3.82^{ab}	3.17^{b}	3.54^{b}
Threonine	0.97^{b}	1.23^{a}	1.12^{a}	1.07^{b}	1.06^{b}
Serine	1.03^{d}	1.45^{a}	1.34^{b}	1.21^{c}	1.23^{c}
Glutamic acid	2.17^{c}	2.74^{a}	2.35^{b}	2.39^{b}	2.38^{b}
Proline	2.82^{a}	2.01^{b}	2.18^{b}	1.91^{c}	2.11^{b}
Glycine	0.94^{c}	1.21^{a}	0.97^{c}	1.08^{b}	0.95^{c}
Alanine	1.11^{c}	1.38^{a}	1.16^{c}	1.29^{b}	1.19^{c}
Cysteine	0.09^{a}	0.09^{a}	0.09^{a}	0.07^{a}	0.08^{a}
Valine	1.09^{c}	1.46^{a}	1.27^{b}	1.32^{b}	1.22^{bc}
Methionine	0.04^{b}	0.09^{a}	0.06^{ab}	0.07^{a}	0.03^{b}
Isoleucine	0.88^{c}	1.11^{a}	0.91^{b}	1.06^{a}	0.92^{b}
Leucine	1.51^{bc}	1.91^{a}	1.56^{b}	1.8^{a}	1.61^{b}
Tyrosine	0.68^{b}	0.79^{a}	0.72^{a}	0.73^{a}	0.73^{a}
Phenylalanine	1.11^{b}	1.31^{a}	1.26^{a}	1.24^{ab}	1.16^{b}
Total	20.13^{c}	25.07^{a}	22.42^{b}	21.88^{bc}	21.76^{bc}

The different letters (a, b, c) after the average show statistically significant differences between the analyzed variants.

In both alfalfa varieties, the content of aspartic acid and the irreplaceable amino acid valine is significantly increased. Aspartic acid is a starting substrate in the biosynthesis of the essential amino acids lysine, threonine, isoleucine and methionine, as well as asparagine, directly related to the activity of aspartic acid. In addition, asparagine is one of the transport forms of amino acids due to the low C:N ratio in its molecule. If we compare this data with the results of the effect of the tested products on the efficiency of nitrogen fixation (unpublished data), the logic behind this is: Tecamin Max increases the activity of the enzymes related to the efficiency of nitrogen fixation in the tested varieties of alfalfa. As a result, larger amounts of glutamine and asparagine, respectively glutamic and aspartic amino acids, are obtained, as our results show. The trends presented here give rise to the following summary: TecaminMax treatment improves the biological value of proteins - increases the total amount of essential amino acids and changes the ratio of essential amino acids to other proteinogenic amino acids in favor of the essential ones.

As a result of the treatment, there was detected a difference between varieties regarding the amount of proteinogenic amino acids. At Legend variety, the highest amount of proteinogenic amino acids was when treated with Tecamin Max, and at Mnogolistna 1 variety, when Fertigrein Foliar was applied.

Mnogolistna 1 variety has higher crude protein content in the biomass compared to Legend variety in all studied variants. The tendency of

protein increase is kept in all swats. The crude protein content at Legend variety is highest when Tecamin Max is applied (21.65, 21.92 and 21.02% by weight of dry matter, respectively, for first, second and third swats). At Mnogolistna 1 variety, the highest protein content is after Fertigrein Foliar treatment in all three swaths was 23.86, 22.23 and 23.89% to absolutely dry matter (Table 5).

Cellulose content data by swaths shows that Fertigrein Foliar treatment influences this indicator, which is kept at all three swaths at Mnogolistna 1 variety.

As a result of this research, it is clear that the use of leaf products can significantly increase the content of irreplaceable proteinogenic amino acids as well as the quality of the protein in the alfalfa biomass. The product containing molybdenum and cobalt (Fertigrain Foliar) has the most significant effect in increasing the amount of amino acids and protein in the biomass. This can be explained by its stimulating effect on the enzymes of nitrogen metabolism – nitrogenase, glutamine synthetase and asparagine synthetase (unpublished data). These results are in agreement with those obtained by other authors working with alfalfa and leaf products (Niewiadomska et al., 2020; Toktarbekova et al., 2020; Niewiadomska et al., 2019; Petkova et al., 2018).

Table 5. Crude protein and cellulose content by swats, average for the experimental period

Variants	1st swat		2nd swat		3rd swat	
	% by weight of dry matter					
Mnogolistna 1	Protein	Cellulose	Protein	Cellulose	Protein	Cellulose
Control	19.58^{c}	22.28^{c}	20.38^{b}	29.39^{bc}	23.12^{a}	33.41^{a}
Tecamin Max	21.94^{b}	25.55^{b}	20.43^{b}	30.84^{b}	23.60^{a}	33.84^{a}
Amino Bore	23.10^{a}	25.96^{b}	21.94^{ab}	31.5^{ab}	23.69^{a}	28.15^{c}
Plantafol	21.76^{b}	27.74^{a}	20.39^{b}	29.94^{b}	23.63^{a}	31.74^{b}
Fertigrein Foliar	23.86^{a}	27.59^{a}	22.23^{a}	32.41^{a}	23.89^{a}	34.27^{a}
Legend						
Control	18.11^{c}	28.08^{a}	20.75^{b}	30.48^{b}	20.20^{b}	35.17^{a}
Tecamin Max	21.65^{a}	27.24^{b}	21.92^{a}	31.76^{a}	22.02^{a}	33.41^{b}
Amino Bore	20.13^{b}	28.77^{a}	21.01^{a}	31.41^{a}	21.67^{a}	35.14^{a}
Plantafol	20.22^{b}	27.14^{b}	21.13^{a}	30.83^{b}	21.19^{ab}	33.91^{b}
Fertigrein Foliar	21.24^{a}	29.31^{a}	21.90^{a}	29.94^{c}	21.86^{a}	32.02^{b}

In the lines, the numbers followed by the identical letters (a, b, c) are not statistically proven.

CONCLUSIONS

The use of Tecamine Max and Fertifrain Foliar significantly increases the total content of proteinogenic amino acids and protein, including essential and nonessential amino acids.

There are variety differences in response of plant reaction to the products. Mnogolistna 1 variety has higher crude protein content in the biomass compared to Legend variety in all studied variants.

All used growth regulators led to a decrease in proline content in both studied varieties. Proline is considered to be a stress indicator amino acid, on the basis of which the applied products can be used at multifolium alfalfa varieties to support the culture to overcome various types of stress.

Cellulose content is also influenced by the treatment - all used products led to an icrease of the researched indicator.

ACKNOWLEDGMENTS

This study was funded by the Research scientific fund, supported by the Agricultural University of Plovdiv.

REFERENCES

Aranjuelo, I., Molero, G., Erice, G., Avice, J., Nogues, S. (2011). Plant physiology and proteomics reveals the leaf response to drought in alfalafa. *Journal of Experimental Botany, 62*, 111–123.

Berova, M., Pevicharova, G., Stoeva, N., Zlatev, Z., Karanatsidis, G. (2013). *Journal of Elementology*, *18*(4), 565–576.

Blumenthal, J., Babyak, M., Doraiswamy, P., Watkins, L., Hoffman, B., Barbour, K., Herman, S., Craighead, W., Brosse, A., Waugh, R., Hinderliter, A., Sherwood, A. (2007). Exercise and pharmacotherapy in the treatment of major depressive disorder. *Psychosomatic Medicine, 69*, 587–596.

Brink, G., Marvin, H., Glen, Sh., Dan, U., Neal, M., Richard, W. (2010). Changes in alfalfa yield and nutritive value within individual harvest periods. *Agronomy journal*, *102*(4), 1274–1282.

Campbell, W. (1999). Nitrate reductase structure, function and regulation: Binding the gap between biochemistry and physiology. *Annual Review of Plant Physiology and Plant Molecular Biology, 50*, 277–303.

Evstigneeva, Z., Gromyko, E., Aseeva K. (1980). Biochemical methods*. M. Science*, 84–86.

Hardy, R., Burus, R. Holsten, R. (1973). Applications of the acetylene-reduction assay for measurement of nitrogen fixation. *Soil Biology and Biochemistry, 5*, 47–81.

Hristozkova, M., Geneva, M., Stancheva, I. (2009). Effect of foliar feeding on nitrogen assimilation in alfalfa plants at insufficient molybdenum supply. *Acta Biologica Hungarica, 60*(2), 211–219.

Hristozkova, M., Geneva, M., Stancheva, I. (2011) Plant Growth and Health Promoting Bactreia. *Microbiology Monographs, 18*, 417–431.

Huset, D.E., Schneble, D.A., Kugler, J.L., and Peterson, M.A. (1991). Registration of 'WL 322 HQ' alfalfa. *Crop Science, 31*, 1699–1670.

Kertikova, D., Yancheva, H. (2000). Variability of some traits in Lucerne within one breeding cycle. *Genetics and Breding, 30*, 1–2.

Kephart, K., Twidwell, E., Bortnem, R. Boe, A. (1992). Alfalfa yield component responses to seeding rate several years after establishment. *Agronomy Journal, 84*, 827–831.

Nedyalkova, K., Petkova, G., Valchovski, H., Donkova, R. (2019). Influence of bio-inoculants (earthworms and nitrogen-fixing bacteria) on rhizosphere microflora of alfalfa. *Pochvoznanie, agrokhimiya i ekologiya/ Bulgarian Journal of Soil Science, Agrochemistry and Ecology*, *53*(1), 27–34.

Niewiadomska, A., Sulewska, H., Wolna-Maruwka, A., Ratajczak, K., Waraczewska, Z., and Budka, A. (2020). The influence of bio-stimulants and foliar fertilizers on yield, plant features, and the level of soil biochemical activity in white lupine (*Lupinus albus* L.) cultivation. *Agronomy*, *10*(1), 150.

Niewiadomska, A., Sulewska, H., Wolna-Maruwka, A., Ratajczak, K., Waraczewska, Z., Budka, A., Głuchowska, K. (2019). The influence of biostimulants and foliar fertilisers on the process of biological nitrogen fixation and the level of soil biochemical activity in soybean (*Glycine max* L.) cultivation. *Applied Ecology and Environmental Research*, *17*(5), 12649–12666.

Peoples, M., Herridge, D., Ladha, J. (1995). Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production. *Plant and Soil*, *174*, 3–28.

Petkova, G., Nedyalkova, K., Perfanova, J., & Eftimova, D. (2018). Effect of Microbial Inoculants on Plant Biomass of Lucerne. *Journal of Balkan Ecology, 21*(4), 370–377.

Popov, N., Nikolov, N., Krastev, A., Mashev, N. (1985). Application of the acetylene method for determination of nitrogenase activity in nitrogen-fixing systems, N. *Proceedings ALL "V. Kolarov", Plovdiv, 30*(3), 51–56.

Radu, F., Ahmadi, M., Cojocariu, I., Marian, F., Bostan, C., Borozan, A. (2010). Genotype-biostimulations interactions in some high quality active principles appearance for alfalfa. *Research journal of agricultural science*, *42*(1), 526–530.

Russelle, M., Birr, A. (2004). Large-scale assessment of symbiotic dinitrogen fixation by crops: soybean and alfalfa in the Mississippi River basin. *Agronomy Journal*, *96*, 1754–1760.

Toktarbekova, S., Meiirman, G., Yerzhanova, S., Abayev, S., Umbetov, A. (2020). Productivity of the green mass of new alfalfa cultivars depending on the effect of macro- and microfertilizers on various phosphorous backgrounds. *Journal of Ecological Engineering*, *21*(2), 57–62.

Wang, J., Zhai, Z., Duan, S., Tian, L., Wang, B. (2003). Effects of DTA – 6 on crude protein and amino acid content of alfalfa (*Medicago sativa* L.). *Journal of China Agricultural University*, *55*(8), 25–28.

Yankov, B., Yancheva, Hr.D. Petkova, D. (1996). Contribution to the selection of multifa ceted alfalfa. *Plant Sciences*, *8*, 15–18.

Zlatev, Z. (2005). Comparative study of water exchange and photosynthesis of different bean genotypes in drought conditions. Thesis.