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EFFECT OF PERSPECTIVE BIOSTIMULANTS ON THE ENZYME ACTIVITIES OF NITROGEN METABOLISM AT ALFALFA IN NEW CLIMATE CONDITIONS

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Abstract

Breeding programs related to alfalfa worldwide are aimed at creating varieties with high productivity, improved quality of green mass, resistance to diseases and pests, durability, rapid recovery after mowing and more. In the face of climate change, it is of great importance to use new, environmentally friendly methods to increase plant adaptability as well as the plant product quality. The role of nitrogen in agricultural production has always been one of the most important priorities of agronomic science, since obtaining good yields in practice is mainly related to providing plants with nitrogen. In this study, we investigated the effect of novel biostimulants on the activity of enzymes from nitrogen metabolism in alfalfa. Their application significantly improves plant metabolism, especially in conditions of drought stress. The results obtained are very important with respect to the green strategy in modern agriculture.

Key words*: alfalfa, biostimulants, climate change, enzyme activity, nitrogen metabolism.*

INTRODUCTION

Breeding programs related to alfalfa worldwide are aimed at creating varieties with high productivity, improved quality of green mass, resistance to diseases and pests, durability, rapid recovery after mowing and more. In the selection of varieties with high quality forage techniques are used to directly influence the protein content, fiber (Huset et al., 1991; Kephart et al., 1992; Petkova et al., 2018; Toktarbekova et al., 2020) or by increasing the percentage of leaves in aboveground biomass. 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 means to improve the adaptability of plants to changing environmental conditions is an important prerequisite for nature conservation in the development of sustainable agriculture. Biostimulants are a variety of chemicals which are applied to plants or soil to improve crop vitality, 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, treatment with phytosanitary products, etc.); facilitate the assimilation of trace elements and the absorption of nutrients; increase the quality of production and more. (Niewiadomska et al., 2019; 2020). The role of nitrogen in agriculture has always been one of the most important priorities of agronomic science, because obtaining good yields in practice is mainly about securing plants with nitrogen. In most plants, its additional application into the soil is necessary, but in legumes the bulk of the element is secured by the biological fixation of atmospheric nitrogen.

The importance of symbiotic nitrogen fixation, which is a complex of interactions between legumes and soil bacteria of the genus Rhizobium, resulting in the formation of specialized formations (root nodes) has been discussed in a number of scientific studies (Peoples et al., 1995; Herridge et al., 2008; Evers, 2011). Nitrogen fixation takes place in the nodes - bacterroids reduce atmospheric molecular nitrogen to ammonia, which is used by plants to synthesize amino acids and proteins. In turn, the plants supply the

bacterroids with carbon to reduce the molecular nitrogen and bring about their growth (Geneva, 2003). The most important are the node bacterias from genus Rhizobium that live in symbiosis with legumes. On average, these bacteria fix from 0 to 500 kg N/ha per year (alfalfa - 500-600 kg N/ha; peas, beans - 50-100 kg N/ha) (Ruselle and Birr, 2004; Kimenov, 1994).

Although node formation is a dynamic process, the size and number of nodes do not fully reflect the degree of symbiotic nitrogen fixation by nodes bacterias and legumes, unless the activity of the enzyme nitrogenesis is directly investigated (EC 1.7.99.2.), which is responsible for carrying out this process (Aranjuelo et al., 2011). The major determinant of the biosynthesis and activity of the nitrogenase are ammonium cations, the end product of nitrogen fixation, which irreversibly inhibit it. Nitrates also suppress the activity of nitrogenase, mainly with the products of its reduction - nitrites, but also with ammonia, which is accumulated as a result of the final reduction of nitrates (Kretovic, 1994). The advantage of legumes is that they can grow on soils with a very low concentration of bound nitrogen, where other plants can not. By increasing mineral nitrogen in the soil (ammonium and nitrate), its absorption from nitrogen-fixing plants increases and symbiotic nitrogen fixation decreases (Campbell, 1999; Ruselle and Birr, 2004).

Nitrogenase activity and nitrogen fixation are highly dependent on photosynthesis and the two processes are directly linked. Nitrate reductase is a key enzyme in regulating nitrate reduction and nitrogen assimilation in crowning plants, including legumes. Nitrate reductase activity in crowning plant cells can be injected by introducing different substrates (Ruselle and Birr, 2004; Hristozkova et al., 2011; Petkova et al., 2018; Nedyalkova et al., 2019).

As a molybdenum-containing enzyme under conditions of molybdenum deficiency, a significant amount of a non-molybdenum precursor of nitrate reductase is continuously synthesized, which is able to rapidly and non-enzymatically attach molybdenum and to form nitrate reductase. Therefore, the formation of a large number of new nitrate reductase molecules is provoked by molybdenum feeding, which enhances nitrogen feeding (Mendel and Haensh, 2002; Kaiser et al., 2005).

In addition to molybdenum, the induction of nitrate reductase activity is provoked 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; Larina et al., 2019).

No matter how they are obtained from the soil, from nitrogen fixation or from nitrate reduction, ammonium cations pass through a specific tunnel of the symbiotic membrane to the host cells, where they are subsequently converted to glutamine. For the legumes from the temperate climate, typical representatives as peas, lupins, alfalfa and more., it is characteristic that they transport the fixed nitrogen in the form of amides (asparagine and glutamine) (Geneva, 2003). The most important enzyme that catalyzes the incorporation of ammonium cations into glutamate by consuming ATP is glutamine synthetase (EC 6.3.1.2).

This enzyme is located in the uninfected part of the nodes. In the bacteride itself, glutamine synthetase exhibits little or no activity (Udvardi and Day, 1997). Its activity is influenced by the presence of Mg^{2+} and Mn^{2+}, but is characterized by different pH optimizers - pH 8 for Mg^{2+} and pH 5 for Mn^{2+} (Kretovic, 1987; Vasileva et al., 2001). Glutamine synthetase exerts a positive effect on the transcription of the genes responsible for the synthesis of nitrogenase, i.e. the formation of the two enzymes in the cell is interrelated, with the appearance of one affecting the biosynthesis of the other.

An alternative route for the incorporation of ammonium ions into node tubers is through the involvement of an enzyme called asparagine synthetase (EC 6.3.5.4), which catalyzes the ATP-dependent synthesis of aspartic acid by aspartic acid and ammonium ions. Which pathways to incorporate ammonium ions will dominate depends on the plant species, its physiological state and environmental conditions.

In unfavorable abiotic factors, various physiological processes, such as

photosynthesis, assimilation of nitrogen, etc., are inhibited. Various techniques are used to overcome adverse conditions, many of which have an unclear mechanism of action (Raza et al., 2019; Niewiadomska et al., 2020). The issue of regulating the biosynthesis, activity, and interaction of nitrogen-fixing enzyme systems in leguminous crops through regulators of these systems (trace elements, phytohormones, and other growth regulators, biostimulants) has not been sufficiently studied. This motivates the present study on the effects of new biostimulants on the activity of enzymes from nitric metabolism in alfalfa.

During 2017-2019 period at the experimental field at the Agricultural university - Plovdiv 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 versions were controlled - treated by Tecamin max, treated by AminoBore, treated by Plantafol, 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. The product promotes the restoration of plants after stressful situations - frost, hail, herbicide effect, phytotoxicity. Provides for the transport in plants of mineral nutrients, including trace elements. Increases plant productivity and yield. Improves product quality.

AminoBore is an organic biostimulant containing 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 NPK 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. Contains amino acids 10%, nitrogen 5%, organic matter 40%, zinc 0.75%, manganese 0.5%, boron 0.1%, iron 0.1%, copper 0.1%, 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.

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 300 ml/ha twice. The products are Tecamine Max, Amino Boron, Plantafol and Fertigrain Foliar.

Samples for enzymatic analyzes (roots with nodes 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.) - 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 (EC 1.6.6.2.). Nitrate reductase 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 acidic acid and with N-(1-naphthyl)- ethylenediamine (Berova et al., 2013).

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 (Dunkan, 1955). Correlation analysis was performed with the SPSS program.

RESULTS AND DISCUSSIONS

The growing conditions of the legumes have a strong influence on the symbiotic nitrogen fixation and the factors that accompany this complex process. At the beginning of our study, a neutral soil profile response was found that was optimal for the flow of nitrogen fixation in alfalfa (Koshkin, 2005). This environment is suitable for the formation of large pink nodes on the roots that develop when there is nitrate deficiency in the soil. The content of mineral nitrogen (NO_3^- and NH_4^+) in our test field is low. These values define soil nitrogen storage as “low storage”. In this case, it is favorable for biological nitrogen fixation, since the increase of the mineral nitrogen in the soil suppresses the activity of the nitrogenase and decreases the part of the "biological" and increases the part of the absorbed nitrogen from the soil. With this low nitrogen stock, it can be considered that the more active growth and biomass accumulation of the treated plants is due primarily to the effects of the products on nitrogen fixation, especially since the soil stock with phosphorus and potassium is very good and promotes effective nitrogen fixation. The same is true for the content and ratio of calcium and magnesium, which is why no additional amounts of nitrogen, phosphorus, potassium, calcium and magnesium have been imported, especially in the conditions of marked droughts that have been observed in the recent years.
In order to establish the relationship between nitrogen fixation activity and the peculiarities of nodes, numerous studies have been carried out on their morphological characteristics, namely: size, volume, weight, quantity, characteristic location on the root system and color. The results are controversial, but larger nodes, predominantly along the main root, pink-colored, are thought to have higher activity. Research data show a higher nitrogen content in these nodes, which confirms the thesis that more active fixation of atmospheric nitrogen by the bacteria present in them (Jimotudis, 2008). Although node formation is a dynamic process, the size and number of nodes do not fully reflect the degree of symbiotic nitrogen fixation by node bacterias and legumes, unless the activity of nitrogenesis is investigated (EC 1.7.99.2.). The results of our studies indicate increased Nitrogenase activity when treated with different products compared to controls in both studied varieties. Comparing the effect of the different preparations can be seen, that Tecamin Max provokes highest Nitrogenase activity at Legend variety and Fertigrein Foliar at Mnogolistna 1 (Figure 1). The assimilation of ammonia, obtained after the atmospheric nitrogen reduction or the reduction of nitrates is carried out with the formation of primary amino acids, amines, atc. In leguminous crops, glutamine and asparagine synthesis reactions catalyzed by glutamine synthetase and asparagine synthetase play a major role in this assimilation. Glutamine synthetase is the main enzyme for the absorption of fixed nitrogen in lucerne plants. It is located outside the cterroid. In the bacteride itself, glutamine synthetase exhibits little or no activity. Treatment of multifolium alfalfa varieties with preparations

containing metallic cations (Mg, Mn, Mo, B, Co) results in a glutamine change - and aspartic synthetases are actively involved in the vegetative mass.

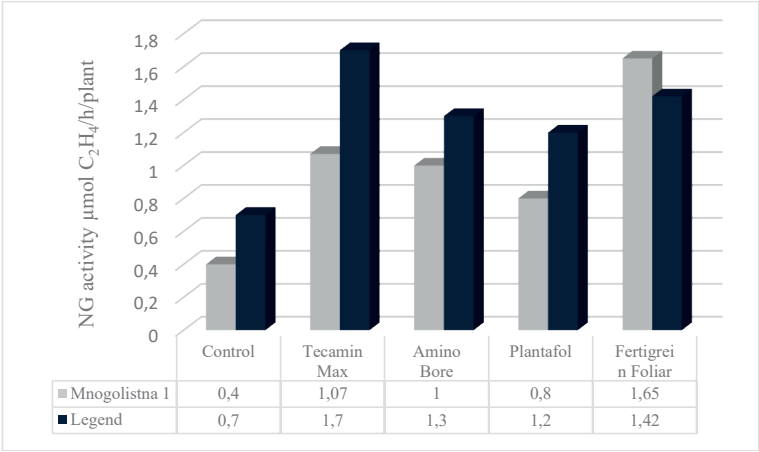

Figure 1. Nitrogenase activity (µmol C_2H_4/h/plant) in alfalfa roots

Glutamine synthetase activity in the untreated plants is highest in the Legend variety. Treatment with the preparation is recommended to show sharply glutamine synthetase activity in both varieties, the highest being in Legend (Figure 2).

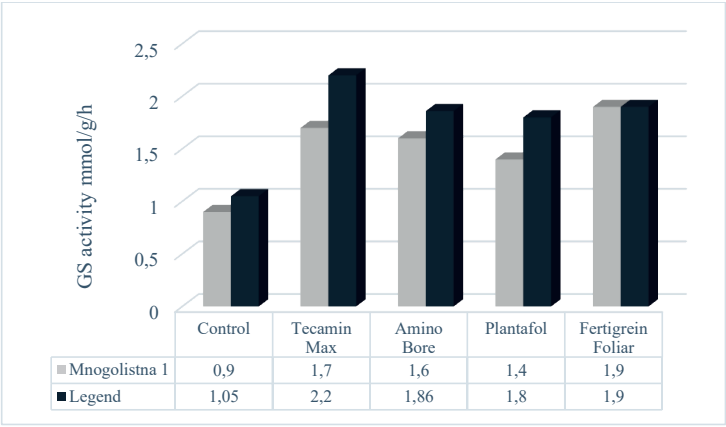

Figure 2. Changes in glutamine synthetase activity (µmol/g/h) in alfalfa roots

Taken separately, both varieties show similar effects after treatment with the preparations. In all variants of treatment, glutamine synthetase activity increased from 157.1 up to 219.9%. This activity is highest when treated with Fertigrein Mnogolistna 1 variety, and with Legend when treated with Tecamin Max. The results for asparagine synthase activity are similar as the one at glutamine synthase. At Mnogolistna 1 variety, again the highest values were observed with FertigreinFoliar treatment, the excess being 277.8% over the control. Treatment with TecaminMax also resulted in an increase of 147.7% over the control variant (Figure 3). Asparagine synthesis activity data at the Legend variety were also unidirectional, with the highest activity when treated with Tecamin Max (205% compared to the control).

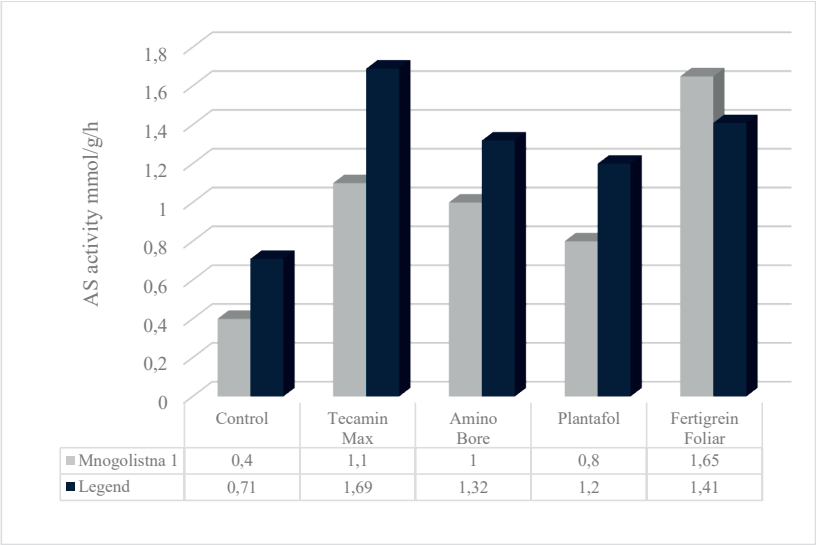

Figure 3. Changes in asparagine synthetase activity (μmol/g/h) in alfalfa roots

The nitrates absorbed by the plants are reduced further in the roots and then in the leaves. Leguminous plants assimilate nitrates mainly through the roots. They contain an energy-dependent system with high affinity for nitrate absorption. Nitrate reductase activity in leaves varies differently from nitrogenase, glutamine synthetase and asparagine synthetase activity. It is significantly reduced when treated with the preparations to almost the same extent in both varieties. The lowest Nitrate reductase activity is reported at Mnogolistna 1, treated by Fertigrain Foliar (Figure 4). Although we are unable to account for the amount of ammonia involved in glutamine synthetase and asparagine synthetase for nitrogen fixation and for nitrate reductase activity and related nitrogen from the soil, the results show that glutamine synthetase activity is high, where the proportion of symbiotic nitrogen is higher. The preparations exert their stimulating effect on the activation of the nitrogenase activity in comparison with the nitrate reductase, which indicates that the treatment primarily stimulates nitrogen fixation. On the other hand, the preparations increase the activity of nitrogenase and glutamine synthetase, the two most important enzymes of nitrogen fixation. Similar results were obtained by Hristozkova et al. (2009) in the treatment of alfalfa with Agroleaf®. With these data, we support the hypothesis of the interconnection between glutamine synthetase and nitrogenase, i.e. the formation of both enzymes in the cell is related - the appearance of a higher activity of one influences the biosynthesis and activity of the other (Kretovic, 1994; Kaiser et al., 2005; Aranjuelo et al., 2011). According to a number of authors (Kretovic, 1994; Kaiser et al., 2005; Aranjuelo et al., 2011) nitrate reductase is inhibited by glutamine, asparagine and other amino acids. Our data support this hypothesis with increased glutamine- and asparagine synthetase activity after treatment with these preparations. With the results obtained, we confirm the hypothesis for the interaction between glutamine synthetase and nitrogenase - the formation of both enzymes in the cell is related - higher activity of one influences the biosynthesis and activity of the other (Kretovic, 1994; Kaiser et al., 2005; Aranjuelo et al., 2011). According to a number of authors (Kretovic, 1994; Kaiser et al., 2005; Aranjuelo et al., 2011), nitrate reductase activity is inhibited by glutamine, asparagine and other amino acids. Our data show increased glutamine- and asparagine synthetase activity after treatment with these preparations.

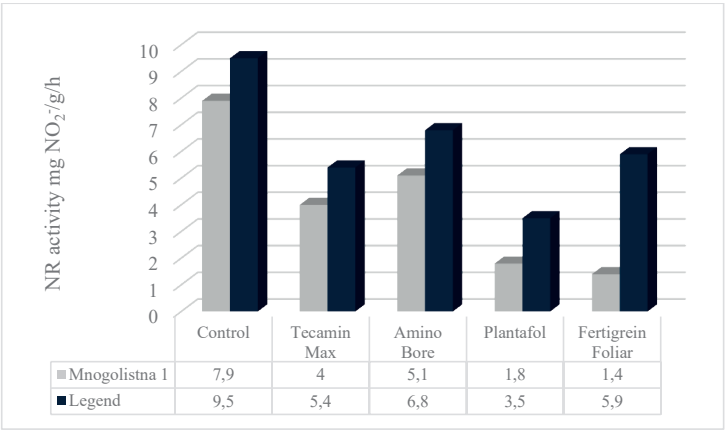

Figure 4. Nitrate reductase activity changes (μg NO_2^-/g/h) at multifoliumf alfalfa varieties

CONCLUSIONS

If the effect of the products, applied to both alfalfa varieties on the activity of the main enzyme systems in the stages of active nitrogen fixation are systematized, the following effects can be considered:

Increase in nitrogenase, glutamine synthetase and asparagine synthetase activity after treatment with Tecamin Max, Amino Bore, Plantafol and Fertigrain Foliar.

Positive dependency between nitrogenase, glutamine synthetase and asparagine synthetase activity.

Variety difference in the effect of the tested products, namely: Tecamin Max increases the nitrogenase, glutamine synthetase and asparagine synthetase activities to a higher degree than the other products in the Legend variety, and Fertigrein Foliar has the highest effect at Mnogolistna 1 variety.

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