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EFFECT OF MINERAL FERTILIZATION ON NITROGENASE ACTIVITY, YIELD, NITROGEN CONTENT AND UPTAKE WITH ALFALFA (*MEDICAGO SATIVA* L.) YIELD*

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Abstract

The aim of the research was to determine the effect of constant mineral fertilization with NPK and diversified fertilization with Fe and Mo microelements on the nitrogenase activity, alfalfa yield and nitrogen content and uptake with yield. A three-year (2012-2014) field experiment was carried out on research fields owned by the Siedlce University of Natural Sciences and Humanities, Poland. The experiment was set up in a completely randomized design with four replications. The first factor consisted of fertilization treatments: control, NPK, $NPKFe_1$, $NPKMo_1$, $NPKFe_1Mo_1$, $NPKFe_2$, $NPKMo_2$, $NPKFe_2Mo_2$. The second factor was composed of the years of research (first, second and third year). Nitrogenase activity in the soil samples was determined. The dry matter yield and total nitrogen content in alfalfa were determined and the nitrogen uptake with yield was calculated. Statistical calculations demonstrated a significant effect of the examined factors on the model amount of nitrogen fixed by *Rhizobium meliloti* bacteria living in symbiosis with alfalfa and free-living bacteria. The significantly highest mean amount of nitrogen was calculated for alfalfa fertilized with $NPKFe_1Mo_1$ (618 kg ha^{-1}). The calculated model amounts of fixed nitrogen were by an average 4.5% higher than the values describing the nitrogen uptake with alfalfa dry matter yield. The significantly highest activity of nitrogenase was found in the soil fertilized with $NPKFe_1Mo_1$ (20-22-124.5-0.5-0.5 kg ha^{-1}) collected in September 2012 (563.5 nM C_2H_4). The research demonstrated that the fertilization of alfalfa with $NPKFe_1Mo_1$ is optimal to achieve the best parameters of the analysed characteristics (except the nitrogen content).

Keywords: Fabaceae, iron, molybdenum, enzymatic activity, N_2 fixed.

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INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a high protein forage plant living in symbiosis with the nodule-colonizing bacteria of the *Rhizobium* genus, capable of assimilating free nitrogen from the air and transferring it to plants (HALBLEIB, LUDDEN 2000, HARDASON, ATKINS 2003). Therefore, nitrogen from the biological reduction process is supplied not only to plants that live in symbiosis with papillary bacteria but also, partly, to the consecutive plants (TA, FARIS 1987). Nitrogen bound in symbiotic systems accounts for about 70 to 80% (PEOPLES, CRASWELL 1992, JONES et al. 2007).

Alfalfa is considered to be one of the most valuable high-protein forage crop (IANNUCCI et al. 2002). It can be used for direct feeding, as green feed, hay, haylage and as dry fodder (BRUMMER et al. 2002). Post-harvest residues or whole legumes treated as green manure increase the nitrogen content in soil (FOWLER et al. 2004). According TALAMUCCI (1994) in alfalfa western and southern Europe is seeded on fields temporarily set aside.

Mineral fertilization of leguminous plants significantly differentiates the enzymatic activity of soil (SYMANOWICZ et al. 2014) as well as the quality and quantity of the yield. Iron and molybdenum are microelements of significant importance for the process of biological reduction of molecular nitrogen (RUTKOWSKA et al. 2017). It is important to monitor the iron content during the cultivation of alfalfa because iron turns into inaccessible forms in soils with a higher pH and high calcium content. Iron is a component of nitrate(III) reductase and nitrogenase. SYMANOWICZ, KALEMBASA (2012) demonstrated a significant effect of iron and molybdenum on the content of nitrogen biologically reduced by Eastern galega (*Galega orientalis* L.). The average molybdenum content in leguminous plants amounts to 0.02 - 3.56 mg kg^{-1} and confirms the process of N_2 fixing by root-nodule bacteria (KALEMBASA, SYMANOWICZ 2009). Alfalfa is a plant susceptible to molybdenum deficiency. Nitrogenase is the most important enzyme catalyzing the direct reduction of molecular nitrogen (HARDASON, ATKINS 2003, GALLOWAY et al. 2008). It consists of two protein complexes. The protein containing Mo-Fe is an enzyme reducing N_2, while the protein containing only Fe provides electrons necessary for the reduction process. The activity of nitrogenase is an indicator of the biological reduction of N_2. *Rhizobium meliloti* bacteria located on alfalfa roots can fix 150 to 350 kg nitrogen per hectare (MARTYNIUK 2012). In the research by RADKOWSKI, GRYGIERZEC (2006) which compared the yielding and protein content in selected varieties of alfalfa and hybrid alfalfa, varied amounts of nitrogen were found (between 2.72% and 3.52%). The nitrogen content in the dry matter of alfalfa depends on the time of harvest and development phase. PIETRZAK (2011) found that alfalfa harvested as the first crop contained 43 g kg^{-1} nitrogen (before budding) and 22 g kg^{-1} nitrogen (end of flowering). In subsequent crops, the nitrogen content determined in dry matter was between 24 and 35 g kg^{-1}.

The aim of the research was to determine the effect of constant mineral fertilization with NPK and diversified fertilization with Fe and Mo microelements on the nitrogenase activity, alfalfa yield and nitrogen content and uptake with yield.

MATERIAL AND METHODS

Research site and experimental design

A three-year (2012-2014) field experiment was carried out in on research fields owned by the Siedlce University of Natural Sciences and Humanities, Poland (52°17′ N, 22°28′ E). The experiment was performed in a completely randomized design with four replications composed of two factors. The first factor comprised: the control, NPK, $NPKFe_1$, $NPKMo_1$, $NPKFe_1Mo_1$, $NPKFe_2$, $NPKMo_2$, $NPKFe_2Mo_2$ fertilization regimes. The second factor corresponded to the years of research (1st, 2nd, 3rd). Mineral fertilization supplied (in kg ha^{-1}): N – 20, P – 22, and K – 124.5. Nitrogen was applied in the early spring as 34% ammonium nitrate, phosphorus – in autumn as 46% triple superphosphate, potassium – as 60% potassium salt in two doses (I – in early spring at 80 kg ha^{-1}; II – after the first swath at 64.5 kg ha^{-1}). Fertilization with Mo and Fe as $FeSO_4 \cdot 7H_2O$ (20.2% Fe) and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (54.3% Mo) was split into two doses: 0.5 and 1.0 kg ha^{-1}. Experimental plots were 3 m^2 in area. Alfalfa cv. *Verko* in a dose of 30 kg ha^{-1} was sown on the optimal agrotechnical date in 2012 (20 April). Bacterial vaccine Nitragina containing *Rhizobium meliloti* was introduced to soil together with the seeds. According to the IUSS World Reference of Soil Resources (2014), the soil on which the field experiment was carried out was classified as *Hortic Anthrosol*. It had an optimum chemical composition for the growth and development of the plant (Table 1).

Sampling and analyses

Soil samples were collected from each fertilizer treatment after harvesting alfalfa on subsequent dates (August 2012, September 2012, June 2013, July 2014). Soil samples were taken from a depth of 0-30 cm. Soil nitroge-

Table 1

Chemical characteristics of experimental soil

Humus horizon	pH_{KCl}	pH_{CaCl2}	$N\text{-}NH_4$*	$N\text{-}NO_3$*	$N_{tot.}$	$C_{org.}$	P*	K*	Mg*
			(mg kg^{-1})		(g kg^{-1})		(mg kg^{-1})		
Ap	6.82	7.01	22	270	2.2	36.4	240	81	95

* available forms

nase activity was measured by an acetylene to ethylene reduction assay, using a CSI gas chromatographer with an FID (flame ionization detector) in the Department of Agricultural Microbiology of the Institute of Soil Science and Plant Cultivation – State Research Institute in Puławy (Poland). During the growing season, alfalfa was harvested four times in the budding phase. The dry matter yield was determined. Then averaged samples were prepared (dried and ground). The total nitrogen content in alfalfa was determined on a Perkin Elmer (Waltham, USA) CHNS/O 2400 autoanalyzer coupled with a thermal conductivity detector (TCD) and using acetanilide as the reference material, and results served to calculate the nitrogen uptake with alfalfa yield. A model amount of nitrogen bound by the bacteria *Rhizobium meliloti* and slow-growing bacteria was also calculated.

In determination of the model amount of nitrogen fixed by the biomass of alfalfa, a modified equation given by Høgh-Jensen et al. (2004) was applied. The balance of N_2 fixed by the alfalfa biomass according to the authors was as follows:

$$N = N_1 \cdot N_2 (1 + N_3 + N_4),$$

where:

N – model amount of nitrogen fixed by the alfalfa biomass (kg ha^{-1});

N_1 – nitrogen amount collected with the dry matter yield of alfalfa (kg ha^{-1});

N_2 – empirical coefficient (share of fixed nitrogen in the amount of nitrogen contained in the dry matter of the aerial part of alfalfa). The value of this coefficient is 0.75 for alfalfa (Høgh-Jensen, Schjørring 1997), and for 0.6966 for applying mineral nitrogen fertilizers (20 kg ha^{-1}), $N_2 = 0.75 - (20 \cdot 0.00267)$;

N_3 – empirical coefficient (share of fixed nitrogen in roots and stubble in the total amount of fixed nitrogen in the aerial part of alfalfa). For the species under examination, it is 0.25 (Pietrzak 2011);

N_4 – empirical coefficient (share of fixed nitrogen immobilized in soil organic matter in the total amount of fixed nitrogen in the aboveground part of alfalfa). For alfalfa, it equals 0.25 (Høgh-Jensen, Schjørring 2001).

Meteorological conditions

Characteristics of hydrometeorological conditions were provided by the Institute of Meteorology and Water Management in Warsaw, the Hydrological and Meteorological Station in Siedlce (Table 2). In the years of research, average temperatures in the growing seasons coincided with the average values of temperatures in individual months for many years. The months with the highest average temperatures were July (19.9°C average) and August (average 18.0°C). The lowest temperatures in the growing seasons were recorded in April (average 8.7°C). An analysis of the sums of atmospheric precipitation showed that they were significantly different in particular vegetation periods. The lowest amount of rainfall was recorded in 2012

Table 2

Characteristics of hydrometeorological conditions, on the basis of data provided by the Institute of Meteorology and Water Management in Warsaw, the Hydrological and Meteorological Station in Siedlce

Research years	Months							
	Apr	May	June	July	Aug	Sept	Oct	mean / sum
Temperature (°C)								
2012	9.0	14.5	16.4	20.4	18.0	14.2	7.5	14.3
2013	7.5	15.3	17.7	18.8	18.3	11.4	9.6	14.1
2014	9.7	13.7	15.1	20.5	17.8	13.7	8.4	14.1
Mean	8.7	14.5	16.4	19.9	18.0	13.1	8.5	14.1
Mean for multiyears 1995 - 2011	8.9	13.7	16.8	19.3	18.0	13.2	8.1	14.0
Rainfalls (mm)								
2012	40.3	59.7	118.7	41.4	64.1	30.8	41.6	396.6
2013	57.6	145.8	111.9	49.1	44.1	86.6	18.0	513.1
2014	39.5	79.5	74.2	37.5	105.7	26.3	3.0	365.7
Mean	45.8	95.0	101.6	42.7	71.3	47.9	20.9	425.2
Mean for multiyears 1995 - 2011	31.7	56.6	65.4	81.3	76.6	52.3	30.4	394.3

(average 543.7 mm), and the highest one was in 2013 (average 682.4 mm). Average values of precipitation in the years of the experiment were lower than the average rainfall for many years (average 765.0 mm).

Statistical analysis

The results were statistically analyzed using the analysis of variance for a two-factor experiment, available in the ANOVA program. The least significant differences (LSD) were determined using the Tukey's test at the significance level of $p \leq 0.05$. The linear regression equations and correlation coefficients between selected features were determined (Statistica 12 PL, Statsoft 2017).

RESULTS AND DISCUSSION

The statistical analysis demonstrated significant differences in the average dry matter yield of alfalfa under the effect of fertilization applied and in different years of research (Table 3). The significantly highest mean dry matter yield of 16.98 Mg ha^{-1} was obtained from the $NPKFe_1Mo_1$ fertilization treatment. It was higher by 13.69 Mg ha^{-1} than the control. A significant

Table 3

Yield of dry matter of alfalfa (Mg ha^{-1})

Fertilization treatments	Year			Mean
	2012	2013	2014	
Control*	6.34	2.37	1.16	3.29
NPK	10.79	14.59	20.70	15.36
$NPKFe_1$	10.35	13.96	19.76	14.69
$NPKMo_1$	9.35	14.82	22.01	15.39
$NPKFe_1Mo_1$	11.81	17.41	21.73	16.98
$NPKFe_2$	11.21	14.92	21.59	15.91
$NPKMo_2$	13.00	14.86	20.98	16.28
$NPKFe_2Mo_2$	11.21	14.94	18.76	14.97
Mean	10.51	13.48	18.34	14.11
$LSD_{0.05}$ for: fertilization – 8.03, years – 3.65, interaction – n.s.				

* control – 0, N – 20, P – 22, K – 124.5, Fe_1 – 0.5, Mo_1 – 0.5, Fe_2 – 1.0, Mo_2 – 1.0 (kg ha^{-1})

increase in the mean dry matter yield of the test plant (by 74.5%) was observed between the first and the third year of the research. The significant increase in yields in the third year resulted from the more intensive process of the biological reduction of molecular nitrogen, which had an impact on the growth of alfalfa biomass. The yield of dry matter of alfalfa in the first year was between 8.88-11.7 Mg ha^{-1}, and was similar to the yield obtained by Andrzejewska et al. (2015).

After three years of the experiment, no significant impact of the examined factors or their combined effects on the nitrogen content in alfalfa was found (Table 4). According to the mean values from the three years, the highest nitrogen content (36.85 g kg^{-1}) was obtained in alfalfa collected from plots fertilized with $NPKFe_2$. The highest nitrogen content was determined in samples of the plant collected in the first year of the field experiment (36.40 g kg^{-1}), when it was higher than the three-year mean nitrogen content by 3.5%.

Fertilization and years of the research significantly differentiated the mean nitrogen uptake with alfalfa yield (Table 5). The mean uptake of nitrogen with alfalfa yield in each fertilization treatment was higher than the nitrogen uptake by the control plants. The highest mean nitrogen content from the three years of the research was recorded in the $NPKFe_1Mo_1$ fertilization treatment. Nitrogen uptake with yield increased in the consecutive years of the research from 382 kg ha^{-1} in the first year to 649 kg ha^{-1} in the third year. The nitrogen uptake with alfalfa yield was correlated with the dry matter yield of alfalfa (mean nitrogen uptake = 4.12 + 35.29x; r = 0.99). The increase in the dry matter yield of alfalfa in the subsequent years resulted in an increased nitrogen uptake with yield. In the first year

Table 4

Content of nitrogen in alfalfa (g kg^{-1} DM)

Fertilization treatments	Year			Mean
	2012	2013	2014	
Control*	36.56	35.05	36.43	36.01
NPK	36.45	36.33	35.95	36.24
$NPKFe_1$	36.39	34.54	35.08	35.33
$NPKMo_1$	35.98	33.23	34.76	34.65
$NPKFe_1Mo_1$	36.76	33.68	34.81	35.08
$NPKFe_2$	37.59	36.61	36.36	36.85
$NPKMo_2$	36.43	35.51	34.14	35.36
$NPKFe_2Mo_2$	35.04	36.48	36.95	36.15
Mean	36.40	35.18	35.56	35.71
$LSD_{0.05}$ for: fertilization – n.s., years – n.s., interaction – n.s.				

* explanations as under Table 3

Table 5

Nitrogen uptake with alfalfa yield (kg ha^{-1})

Fertilization treatments	Year			Mean
	2012	2013	2014	
Control*	232	83	42	119
NPK	393	530	744	556
$NPKFe_1$	377	482	693	517
$NPKMo_1$	336	492	765	531
$NPKFe_1Mo_1$	434	586	756	592
$NPKFe_2$	421	546	785	584
$NPKMo_2$	474	528	716	573
$NPKFe_2Mo_2$	393	545	693	544
Mean	382	474	649	502
$LSD_{0.05}$ for: fertilization – 280, years – 127, interaction – n.s.				

* explanations as under Table 3

of the research, the nitrogen uptake (N_u) was correlated with the dry matter yield ($N_u = -2.19 + 36.62x$; $r = 0.99$). The correlation was as follows in the second ($N_u = 5.02 + 34.78x$; $r = 0.99$) and in the third year ($N_u = 4.75 + 35.15x$; $r = 0.99$). The pot tests carried out by WIERZBOWSKA, ŻUK-GOŁASZEWSKA (2014) showed that inoculation of fenugreek seeds of *Rhizobium meliloti* and applied nitrogen fertilization caused an increase in the phosphorus content of seeds, while nitrogen fertilization significantly increased the protein content of seeds.

The intensity of the biological reduction of molecular nitrogen (N_2) was assessed on the basis of measurements of the intensity of the reduction of acetylene to ethylene. The research conducted on nitrogenase demonstrated its ability to reduce gas, including acetylene. The statistical analysis demonstrated a significant effect of the mineral fertilization and dates of collecting soil samples and their combined effect on nitrogenase incubated for 48 h with C_2H_2 (Table 6). The effect of the dates for collecting soil samples on

Table 6

Nitrogenase activity in soil incubated for 48 h with C_2H_2 (nM C_2H_4)

Fertilization treatments	Terms				Mean
	August 2012	September 2012	June 2013	July 2014	
Control*	366.7	311.5	256.9	147.1	270.5
NPK	240.0	236.8	284.8	171.4	233.2
$NPKFe_1$	436.3	332.4	442.2	129.4	235.1
$NPKMo_1$	214.4	310.9	242.6	219.2	246.8
$NPKFe_1Mo_1$	308.2	563.5	425.9	201.2	374.7
$NPKFe_2$	394.2	317.5	419.2	176.5	326.8
$NPKMo_2$	430.7	303.7	342.2	167.7	311.1
$NPKFe_2Mo_2$	281.0	364.2	306.2	193.3	286.2
Mean	333.9	342.6	340.0	175.7	298.0
$LSD_{0.05}$ for: fertilization – 38.9, dates – 23.2, interaction T/F – 65.6, interaction F/T – 77.9					

* explanations as under Table 3

nitrogenase activity should be associated with the soil sample collection depth and weather conditions (Streeter 1994, Martyniuk et al. 2005). The significantly highest mean activity of nitrogenase was found in soil fertilized with $NPKFe_1Mo_1$ (374.7 nM C_2H_4), where it was by 37% higher than the activity of nitrogenase determined in soil from the control. In the soil collected in August (2012), September (2012) and June (2013), the average activity of nitrogenase was determined on a similar level (means 333.9, 342.6 and 340.0 nM C_2H_4). The significantly lowest activity of nitrogenase was found in soil collected in July 2014 (175.7 nM C_2H_4). The reduced nitrogenase activity in July 2014 was mainly related to the depth of soil sampling. The soil was taken from a depth of 30 cm and the root system of alfalfa reached 60-90 cm deep into soil. The soil temperature at the depth of 90 cm (above 20°C) could also have had an indirect influence. Optimal soil temperature for nitrogenase activity is 15-20°C. The significantly highest activity of nitrogenase was found in soil fertilized with $NPKFe_1Mo_1$ (20-22-124.5-0.5--0.5 kg ha^{-1}) collected in September 2012 (563.5 nM C_2H_4). These results were supported by the findings of Unkovich, Pate (2000), who indicated the need for Fe fertilization, since iron is included in nitrogenase proteins (Fe protein and MoFe protein). The research conducted by Mocek-Płóciniak et al. (2008)

demonstrated that the cultivation of alfalfa inoculated with 10% *Sinorhizobium meliloti* Bp in soil pH of 6.5 resulted in the highest nitrogenase activity, more abundant fresh matter and a very well developed root system.

Statistical calculations demonstrated a significant effect of the examined factors on the model amount of nitrogen fixed by *Rhizobium meliloti* bacteria living in symbiosis with alfalfa and free-living bacteria (Table 7). The significantly highest mean amount of nitrogen was calculated for alfalfa fertilized with $NPKFe_1Mo_1$ (618 kg ha^{-1}). It was five times higher in comparison to the amount determined for the test plants from the control treatment. The calculated model amounts of fixed nitrogen were 4.5% higher than the values describing nitrogen uptake with alfalfa dry matter yield. The results obtained from the model calculations (Table 7) indicate that the use of mineral nitrogen in a dose of 20 kg ha^{-1} before mowing the grass is unnecessary, especially in consecutive years. The average actual nitrogen uptake with alfalfa yield in the subsequent years of the study was lower by 18-22-31 kg ha^{-1} (Table 5). The model amount of nitrogen biologically fixed by *Rhizobium* and free-living bacteria was correlated with the nitrogenase activity in the soil collected on the subsequent dates (years of research). The increase in the nitrogenase activity in the analysed soil samples led to an increase in the amount of biologically reduced N_2 (August 2012, $N_2 = 342.9 + 0.17x$; September 2012, $N_2 = 332.9 + 0.19x$; June 2013, $N_2 = 165.5 + 0.97x$; July 2014, $N_2 = -38.7 + 4.08x$).

Table 7

Model amount of nitrogen fixed by *Rhizobium meliloti* bacteria living in symbiosis with alfalfa and free-living bacteria (kg ha^{-1})

Fertilization treatments	Year			Mean
	2012	2013	2014	
Control*	242	87	44	124
NPK	411	554	777	581
$NPKFe_1$	394	504	724	541
$NPKMo_1$	351	514	799	555
$NPKFe_1Mo_1$	453	612	790	618
$NPKFe_2$	440	570	820	610
$NPKMo_2$	495	552	748	598
$NPKFe_2Mo_2$	411	569	724	568
Mean	400	495	678	524
$LSD_{0.05}$ for: fertilization – 292, years – 133				

* explanations as under Table 3

CONCLUSIONS

1. The highest yield of dry matter of alfalfa (average 18.34 Mg ha^{-1}) was harvested in the third year of the research.

2. The significantly highest activity of nitrogenase was found in soil fertilized with $NPKFe_1Mo_1$ (20-22-124.5-0.5-0.5 kg ha^{-1}), collected in September 2012 (563.5 nM C_2H_4).

3. The highest amount of nitrogen fixed by *Rhizobium meliloti* and free-living bacteria (618 kg ha^{-1}) was calculated for alfalfa fertilized with $NPKFe_1 Mo_1$.

4. The research demonstrated that fertilization of alfalfa with $NPKFe_1Mo_1$ is optimal for obtaining the best values of the analyzed parameters (except the nitrogen content).

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