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Professional paper

EFFECTS OF AGROCHEMICAL PROPERTIES, FERTILIZATION AND GROWTH STAGE ON THE CONTENTS OF NITRATES AND THE NITRATE REDUCTASE ACTIVITY IN ALFALFA LEAVES

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Abstract

Alfalfa (Banat ZMS II) was grown in vegetation pots with 12 kg of air-dry soil from different localities with known contents of total nitrogen, phosphorus, potassium, and molybdenum. The content of the same nutrients is also known in the dry mass of the experimental alfalfa.

The measured nitrates showed a dependence on the content of N, P, K and Mo in the soil. In the unfertilized plants, in the pre-flowering phase, a higher nitrate content was measured in the leaves of the Jegunovce and Raduša localities (16.01; 16.10 µmolNO_3- g/FW^{-1}) that is, of the fertilizer plants in the leaves of Saraj and Raduša (22.12; 19.58 µmolNO_3- g/FW^{-1}). In the flowering phase, the average nitrate content in the unfertilized ones was lower by 26.1%, and in the fertilized plants by 33.54%.

During before flowering phase, higher nitrate reductase activity (in vivo) was measured in fresh leaf mass from the Jegunovce and Raduša localities (614.39; 605.41 nmol NO_2- g/FW^{-1}/h^{-1}), while lower in the leaves of Saraj (529.84 nmol NO_2- g/FW^{-1}/h^{-1}). Under in vitro conditions, higher activity in the leaves of Jegunovce (728.10 nmol NO_2- g/FW^{-1}/h^{-1}), and lower in those of Kodzilari (560.29 nmol NO_2- g/FW^{-1}/h^{-1}).

In the flowering phase, the reduced enzyme activity by an average of 56.29% is accompanied by a decrease in the amount of nitrates and the mentioned biogenic elements in the dry mass of the leaves.

Keywords: soil, alfalfa, leaves, nitrates, nitrate-reductase, phase of development, fertilization.

1. Introduction

Alfalfa (*Medicago sativa L.*) is a nutritionally rich perennial legume that is widely used in different livestock production systems worldwide (8,14). Studies conducted on alfalfa indicate its high nutritional value, primarily due to the abundance of crude protein and rich mineral composition.

Research in the field of mineral nutrition of alfalfa (38,8,20) indicates the influence on its growth, development, and qualitative composition. The application of high amounts of nitrogen fertilizers tends to suppress root nodulation when phosphorus levels are low, while high phosphorus availability can stimulate nodulation even with significant nitrogen application (5, 36). High rates of K fertilization in “spring” alfalfa (*Medicago saliva L.*) increased the number of nodules, populated by nitrogen-fixing bacteria - *Rhisobium sp*.(17), enzyme activity, yield and N_2 fixation as well as phosphorus uptake (12,34).

In addition to the aforementioned symbiosis, alfalfa may still suffer from nitrogen deficiency due to inadequate nutrient levels in the soil (41), salt stress (42) and the influence of water as a factor in the uptake of mineral nutrients (38,27).

Nitrate uptake by bean plants (*Phaseolus vulgaris L.*) is initially slower, followed by a period of intensive uptake (30). Studies on peas (29) have shown that uptake and translocation rates of nitrate ions and organic nitrogen depend on the presence of molybdenum, lead, and cadmium ions in the soil. Studies on alfalfa (37) have also shown that nitrate uptake also depends on temperature, O_2 concentration, and the supply of nitrates to the soil.

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In legumes (*Vicia sp., Pissum sativum, Medicago sativa*), 30-60% of their total nitrate reductase activity is carried out in the root (1). Studies on different varieties of alfalfa indicate the dependence of the qualitative composition of the leaves or hay on the stage of development (7,16,10,14,15). Studies carried out on oilseed rape (33) indicate that during flowering 30-60% of endogenous nitrogen is mobilized from the leaves to the flowers or seeds.

The process of nitrification in spinach leaves, depending on the amount of phosphorus, i.e., the decrease in ATP, indicated the presence of ATP-dependent nitrate reductase activity in spinach leaves (23). Studies on three leafy vegetables determined that with certain increases in nitrate content, nitrate reductase activity does not follow such dynamics, i.e., the presence of a threshold concentration of nitrates, which is why a slowdown of the enzyme activity in alfalfa leaves was observed (6,40). Studies on nitrate uptake and their metabolism (31,19) have shown that when the nitrate reduction capacity of plants, i.e. alfalfa, is exceeded, their accumulation in the shoot occurs.

Studies of nitrate reductase activity have shown a significant dependence on the amounts of nitrates, available potassium, phosphorus and molybdenum in the soil (40,11,24,35)..) as well as from the development phase, i.e. its rapid decrease in the examined leaves (3,4). Research conducted on wheat (35) has also shown the influence of temperature on photochemical reactions and nitrate reductase activity in leaves.

2. Materials and methods

The experiment with alfalfa variety Banat ZMS II was carried out in vegetation pots, filled with 12 kg of air-dry soil, with known content of total nitrogen, phosphorus, potassium, calcium, and magnesium (tab. 1) from 4 localities: Saray (Skopje), Raduscha (Skopje), Yegunovce (Tetovo), and Kodžilari (Veles).

Tab. 1. Agrochemical composition of used soil

Location	Available amounts in the soil				
	mg/100g				
	Total N	P_2O_5	K_2O	CaO	MgO
Saray	7.52	36.02	3.33	526.0	19.0
Yegunovce	7.62	23.17	4.87	622.5	29.3
Raduscha	9.75	28.90	9.89	582.5	195.5
Kodzilari	9.38	50.75	68.20	815.0	63.75

For each site, 20 vegetation pots (a total 80) were set up, each with 10 plants. During the vegetation period, the plants were fed with 7 gr/pot potassium nitrate (KNO3) and 3 gr/pot F-Top NPK (15:30:15 + TE) fertilizer. Four vegetation pots for each site served as controls (without feeding).

In fresh leaves (or frozen in liquid nitrogen) of alfalfa collected during two stages of development, the following was determined: the nitrate content (μg N-NO3) according to the method of Cataldo, 1975 (9). Nitrate reductase activity in vivo (nmol/nitrite/ml) according to the method of Jaworski, 1971 (21) and in vitro according to the method of Hageman & Reed, 1980 (18).

3. Results and discussion

In the second vegetation year (tab.2) in the before-flowering phase (II cutting), the measured nitrate contents in the leaves of the unfed plants ranged from 14.51 µmolNO_3 (Saray) to 16.10 µmol (Yegunovce) and 16.01 µmolNO_3 (Raduša), i.e. in the leaves of the fed alfalfa from 17.43 µmol (Yegunovce) to 22.12 µmolNO_3 g/FW $^{-1}$ (Saray). In the flowering phase, lower nitrate contents were measured, in the unfed ones by 24.4% (11.6 µmol NO_3), i.e. in the fed ones by 33.57 % (12.9 µmol NO3 g FW $^{-1}$). In the before-flowering phase, the higher nitrate contents in the leaves of the Raduša and Jegunovce variants are an expression of the weaker intensity of the nitrate reduction carried out in the roots, primarily due to the lower supply of soils with phosphorus (23.17mg and 28.90mg/100g), which has a positive role in nitrogen metabolism and nodulation (26,36,5,19), or the lower nitrate contents in the leaves of Saray and Kodžilari (14.51 and 14.76 µmol NO_3) due to the better supply of soils, especially with phosphorus, tab. 1 (36.02 and 50.75 mg/100g).

In the before-flowering phase, the higher average nitrate content in the leaves of the fertilized plants of 19.48 µmolNO3g/FW^{-1} compared to the unfertilized ones by 26.98% and their decrease in the flowering phase by 33.57% (12.9 µmolNO3g/FW^{-1}) is a result of the KNO_3 feeding, which has also been established by research by other authors (4,2) and especially the positive influence of added amounts of KNO_3 (17,25,17,34). The higher nitrate content of 22.12 µmol NO3 in Saray leaves is accompanied by a significantly higher content of total nitrogen and potassium in alfalfa leaves (13) i.e. greater sensitivity to applied KNO_3 due to the weaker supply of soil with potassium (Tab.1). with clay because the soil has a lower content of K (3.33 mg/100g), as well as its positive correlation with the higher amounts of available phosphorus (36.02 mg/100g), a ratio also found in previous research (19,8). The lower nitrate content in the leaves, i.e. the weaker uptake of applied KNO_3 in the Yegunovce variant, is primarily due to the good supply of the soil with available calcium (622.5mg/100g) which showed an inhibitory effect of the added KNO_3 (28,36). The different degree of reduction in nitrate content in the flowering phase in the individual variants is primarily a result of the existing difference in the supply of the used soil with available forms of N, K, P, Ca, Mg (Tab.1) i.e. the dependence of nitrate reduction and the amount of nitrates absorbed on their ratio in the soil solution (38,20,13). Also, in the flowering phase, a change in other soil conditions is evident, primarily humidity, temperature conditions, and amount of oxygen, which have an impact on the degree of absorption and mobility of mineral substances (22,41,42,27).

The activity of NR-aza /In vivo (tab.2) in fresh alfalfa leaves showed a significant dependence on the amount of nitrates and the developmental phase. In the before-flowering phase in the unfertilized ones, the activity ranged from 274.8 nmol NO_2 (Kodzhilari) to 310.5 nmol NO_2 (Raduscha), i.e. in the fertilized ones from 529.84 nmol NO_2 (Saray) to 614.39 nmol NO_2 (Yegunovce). In the flowering phase in the fertilized ones, an average decrease in activity of 23.2 % (446.0 nmol NO2) was measured, which was more pronounced in the Raduscha variety (414.60 nmol NO2), i.e. in the unfertilized ones in the Saray variant by 41.5 % (175.50 nmol NO2 /g / FW/h^{-1}).

Tab. 2. Content of nitrates and nitrate reductase activity in alfalfa fresh leaves /in vivo-in vitro/

Location	Stage of gr	Leaves							
		µmol NO3 g FW-1		nmol NO2 g FW-1h-1					
				In vivo				In vitro	
		Unfertilized plants							
			%			% fl./b.fl.	unf./ fert.		% fl./b.fl.
Saray	Before	14.51 ± 0.33		300.0 ± 11.4	100.0			-	- -
Yegunovce		16.01 ± 0.28		304.2 ± 12.7	101.4			-	- -
Raduscha		16.10 ± 0.16		310.5 ± 15.7	103.5			-	- -
Kodzilary		14.76 ± 0.22		274.8 ± 14.4	91.6			-	- -
								-	- -
	Flowering flower	M 15.34	**100.0**	**297.4**		**100.0**	**51.2**		- -
								-	- -
Saray		11.90 ± 0.38	81.9	175.5 ± 5.6	100.0	58.5			- -
Yegunovce		11.11 ± 0.26	69.4	183.8 ± 4.5	104.7	60.4		-	- -

Raduscha		11.21 ± 0.11	69.6	215.7 ± 5.7	122.9	69.5		-	- -	
Kodzilary		12.18 ± 0.16	82.5	190.7 ± 9.2	108.6	69.4		-		
		M 11.60	75.6	**191.5**	-	**64.3**	**42.9**	-		
		Fertilized plants								
Saray		22.12 ± 0.62		529.8 ± 44.5	100.0	100.0		596.9 ± 42.7	100.0	
Yegunovce		17.43 ± 0.42		614.4 ± 32.7	115.9			728.1 ± 37.6	122.1	
Raduscha		19.58 ± 0.27		605.4 ± 24.7	114.3			631.5 ± 32.3	105.8	
Kodzilary		18.78 ± 0.34		573.8 ± 43.2	108.3			560.3 ± 46.5	93.9	
		M 19.48	**100.0**	580.9	-		**100.0**	622.5	-	**100.0**
Saray		13.14 ± 0.30	59.4	442.2 ± 20.6	100.0	83.5		315.1 ± 12.6	100.0	52.8
Yegunovce		11.73 ± 0.37	67.3	434.1 ± 16.4	98.2	70.6		279.1 ± 15.8	88.6	38.3
Raduscha		14.16 ± 0.49	72.4	414.6 ± 11.2	93.8	68.5		240.7 ± 7.7	76.4	38.1
Kodzilary		12.75 ± 0.46	67.9	493.1 ± 43.5	111.5	85.9		265.5 ± 8.7	84.3	47.4
		M 12.94	66.4	446.0	-	**76.8**	**100.0**	275.1	-	44.2

In vivo activity of NR aza (tab. 2) was shown to be dependent primarily on the nitrate content, with higher activity being measured in the Yegunovce variants (614.4 nmol NO_2) and Raduscha (605.4nmol NO_2 g FW $^{-1}$). Within the fertilized plants-leaves, no correct correlation was found between nitrate content and enzyme activity, i.e., in the before-flowering phase in the Yegunovce variant, the amount of nitrate was lower (17.43µmol NO3) in contrast to the higher measured enzyme activity (614.4 nmol NO_2), i.e., in the Saray variant a higher nitrate content (22.12µmol NO3), but lower enzyme activity. This indicates that there is no linear increase in enzyme activity with the amount of nitrate, i.e. there is some threshold of activity in relation to the accumulated nitrate (1,23,6). Lower enzyme activity in the flowering phase is correlated with reduced nitrate content, water, crude protein, and the content of K, P, Mg, and Mo in the leaves, which suffer significant reduction (14,37,40,7).

The measured in vitro enzyme activity performed in the leaves of the fertilized plants (tab.2) in the before-flowering phase showed a significant difference within the experimental variants and they ranged from 560.3 nmol NO_2 /g (Kodžilari) to 728.10 nmol NO_2 (Yegunovce), i.e. an average enzyme activity of 622.46. nmol NO2 /g /FW/h^{-1}. In the flowering phase, the enzyme activity of 275.09 nmol NO_2 was lower by 55.8% compared to the same in the pre-flowering phase. A greater drop in activity at this phase was measured in the Raduscha variant (240.72 nmol NO_2). The rapid decrease in in vitro NR-ase in the flowering phase was not accompanied by a corresponding decrease in nitrates, which were lower by 33.6% (6,32,3).

In vitro enzyme activity in the before-flowering phase, i.e. the observed average of 622.5 nmol NO_2/g/FW compared to the average in vivo activity (580.9 nmol NO_2g/FWh^{-1}) measured at the same stage of development was significantly higher (by 7.2%). The obtained correlation indicates the sensitivity of enzyme activity to multiple environmental factors (27,5,29,23,32,37). Also, in vitro enzyme activity in the flowering phase showed a high decrease in activity (by 55.8%), which is primarily correlated with the reduced nitrate content at this stage, which is known to mobilize endogenous nitrogen from the leaves to the inflorescences or pods (33). The measured values for enzyme activity in the leaves of the experimental variants, similar to those in in vivo, did not show a linear decrease in relation to the measured activity in the before-flowering phase. In the before-flowering phase, significantly higher values for enzyme activity were measured, i.e. the measured average activity of 622.49 nmol NO_2/g/FW i.e. in the flowering phase 275.09 nmol NO_2/g/FW, which confirmed the knowledge about the dependence of enzyme activity on the development phase (3,39) which, according to other research, has shown a significant impact on the overall qualitative value of alfalfa (7,20,34,12). Also, the higher measured activity in the leaves of Yegunovce of 728.10 nmol NO_2 g/FW which was not accompanied by a higher nitrate content in the corresponding variant (17.43µmol NO_3) supports the knowledge about the dependence of activity on other factors, primarily the content of N, K, P, Mo in the leaves, the water content, etc. (17,8,14,24,27).

The measured low nitrate contents and enzyme activity in the leaves support research according to which in leguminous plants 30-60% of nitrification takes place in the roots (36.1).

4. Conclusions

1. The nitrate content in alfalfa leaves is dependent on the supply of sufficient amounts of soil nitrogen sources.
2. The agrochemical composition of the soil has a significant impact on the degree of nitrate absorption, primarily the presence of available macronutrients (N, P, K, Ca, Mg).
3. The activity of NR in leaves was shown to be dependent to a certain extent on the amount of nitrate present, i.e. there is no linear increase in enzyme activity.
4. NR ase activity (in vivo and in vitro) showed significantly higher values in the before-flowering stage.
5. During the flowering phase, there is a decrease in nitrate content and a higher degree of reduction in NR activity.
6. The KNO_3 fertilization of alfalfa should be balanced according to its losses and the content of other mineral nutrients in the soil, otherwise there is a possibility of its accumulation and an impact in terms of reducing the nutritional value.

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