ALFALFA GROWTH ON ACID SOIL AS INFLUENCED BY CALCIFICATION AND *ENSIFER* STRAINS INOCULATION

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Abstract

In the present study, the effect of alfalfa inoculation with two acid tolerant rhizobial strains (*Ensifer medicae* and *Ensifer meliloti*) and lime application, on the growth and nitrogen content of alfalfa (*Medicago sativa* L.) in very acid soil was evaluated (pH_{KCl} 4.4). Inoculation with *E. medicae* and/or *E. meliloti* significantly increased shoot dry weight (SDW) of alfalfa plants compared to the control (without inoculation, fertilization and lime application) as well as total nitrogen uptake (µg/plant). Application of lime and rhizobium together, depending on the lime rate (7 or 15 t/ha of lime, and 15 t/ha of dolomite), increased SDW significantly, compared to the inoculation alone. The highest SDW, three times higher than the control, and doubled compared to inoculation and 7 t/ha of lime, was obtained with inoculation and 15 t/ha of lime application. In this treatment soil pH reached optimal values for alfalfa stand establishment, confirming the importance of adequate soil pH in alfalfa cultivation. Alfalfa can grow in acid soil with acid tolerant rhizobium inoculation alone, but the application of lime is of great significance.

Keywords: rhizobia, *Medicago sativa* (L.), inoculation, yield increase, lime

INTRODUCTION

Biological N_2 fixation represents the major source of N input in agricultural soils (Zahran, 1999). Besides nutrient availability, low soil pH is one of the most limiting factors for plant production. Acid soils take up around one third of the soil worldwide, while in Serbia, around 60% of arable land is of acid reaction (Sikiric et al., 2011). Legumes are generally more sensitive than forage grasses to nutrient deficiencies and low soil pH. Soil pH of 5.5 is considered the lower limit for most forage legumes. In acid soil, plant growth was limited due to toxic concentrations of H, Al, Mn and Fe and deficiency of N, P, Ca, Mg, B and Mo (Von Uexküll and Mutert, 1995). The sensitivity of legume to acid soil is also the result of soil pH influence on the rhizobial bacteria.

Alfalfa (*Medicago sativa* L.) is one of the most important forage crops in many countries due to a high biomass yield, excellent nutritive value and high digestibility. Alfalfa can establish a nitrogen fixing symbiosis with soil bacteria, rhizobia (*Ensifer* (*Sinorhizobium) meliloti*) and fix atmospheric nitrogen of the benefit to the plant. Alfalfa/*E. meliloti* symbiotic association is one of the most efficient interactions between rhizobia and legumes, since it usually fixes 140–210 kg/ha of N per year in the field (Provorov and Tikhonovich, 2003). However, alfalfa is one of the most sensitive legumes to soil acidity, compared to red clover, soybean, etc., requiring a pH above 6.0 and, to begin a stand a pH above 6.5.

Many agricultural regions throughout the world have moderately acidic soils preventing the *Ensifer/Medicago* symbiosis from reaching its full potential (Howieson, 1995). Therefore, many previous works dealt with cultivation of alfalfa in acid soils with lime application and/or inoculation in Serbia and worldwide (Stevović et al., 2001; Milic et al., 2014; Katic et al., 2009; Gomes et al., 2000). Some researches indicated that acid-tolerant isolates belonging to *E. medicae* species can efficiently fix nitrogen in association with both annual acid soil adapted *Medicago* hosts, as well as with the perennial forage legume *M. sativa*, in contrast to *E. meliloti* (Garau et al., 2005).

Previously, we have isolated the strains of *E. medicae*, as well as *E. meliloti* from *M. sativa* which have optimal growth in the pH of 6-9, but they could also grow at 5.5 (Stajković-Srbinović et al., 2012). In addition, these strains also showed good nitrogen-fixing potential with more alfalfa cultivars in soil of mild acid reaction (Delić et al., 2013).

In this work we have investigated the ability of cultivation of *M. sativa* in very acid soil (pH 4.4) with inoculation with *E. medicae* or/and *E. meliloti* and calcification. We have chosen the alfalfa cultivar K-28 (Kruševačka 28) which has increased tolerance to mildly acid soils, so it can grow well in marginally acid soils for alfalfa cultivation.

MATERIALS AND METHODS

To set up the experiment, top soil layer (30 cm) of an acid Pseudogley soil from Varna village (near Valjevo, Serbia), with the following chemical characteristics (Table 1) was used. Each pot was filled with 2 kg of air-dried soil and 20 seeds of alfalfa were planted. The experiment was observed for a year, with 8 different treatments and one control with six replications: inoculation (*E.mediace* strain LR1KS or/and *E. meliloti* L3Si), inoculation and addition of 7 or 15 t/ha of

$CaCO_3$, NPK fertilization without inoculation (NØ), NØ and 15 t/ha of dolomite, control Ø (no inoculation and no fertilization) (Table 2).

Table 1. Soil characteristics

Parameter		Parameter	
pH in KCl	4.4	N_{tot} %	0.139
pH in H_2O	5.8	NH_4^+-N mg/kg	7.25
Humus %	3.37	NO_3^--N mg/kg	12.25
C_{org} %	2.17	P mg/kg	31.07
		K mg/kg	142.10
		Mg mg/kg	250.00
		Ca mg/kg	1.20

Six months before the experiment was set up, 4.67 g/pot of $CaCO_3$ (equivalent to 7 t/h of lime) or 10 g/pot of $CaCO_3$ or $CaMgCO_3$ (equivalent to 15 t/ha of lime or dolomite) were added, mixed thoroughly and watered regularly. NPK mineral fertilization in amount of N 60 kg/ha, P 100 kg/ha and K 100 kg/ha was performed in fertilized treatments.

The pots were kept in a closed greenhouse in semicontrolled conditions and plants were harvested, soil samples were taken and analysed 15 weeks after the experiment was establishment. Roots were carefully removed from the pots, washed free of soil and the root and shoot portions of alfalfa were separated and measured. The shoot and root length were measured. Plant shoots and roots were dried in an oven at 70˚C to constant weight, weighed and the average dry weight per plant was calculated. Total plant N was determined with elemental CNS analyzer. The effect of the treatments was evaluated using analysis of variance (SPSS 16.0 program, 2007), and significant differences between means were tested by Duncan's multiple range test.

RESULTS AND DISCUSION

Previously it was estimated that soil pH range of 6.8 to 7.2 is ideal for new alfalfa seedlings to establish and develop rapidly (http://animalrange.montana.edu). In this study, the application of 7 t/ha of lime increased soil pH significantly up to 6.21, while the optimal soil pH was reached with the application of 15 t/ha of lime or dolomite (pH 7.20 and 7.06 respectively). Soil was at the medium level of total N supply in all treatments, and there were no differences between treatments (Table 2). The N content in soil did not change even after mineral nitrogen fertilization.

The plant height was in the range from 21.00 cm in the control up to 31.68 cm in the treatment with inoculation and 15 t/ha lime application, while only the treatment with NPK fertilization and lime application was significantly different from the control.

Inoculation with *E.medicae* and/or *E.meliloti* increased alfalfa shoot dry weight (SDW) significantly compared to the control (0.072 mg/plant) up to the same level (0.109, 0.115 and 0.111 mg/plant). Although in the treatment with 7 t/ha of lime and inoculation soil pH increased up to 6.21, there was no increase in SDW (0.113 mg/plant) compared to inoculation alone. Previously, it was shown that alfalfa yields drop sharply in the first-cutting when soil pH falls below 6.7, which is also the case in our study (Undersander et al, 2001).

The highest SDW was obtained with inoculation and 15 t/ha of lime application (0.230 mg/plant) when pH increased to 7.21, which is two times higher compared to inoculation and 7 t/ha of lime, and 50% higher compared to inoculation and 15 t/ha of dolomite. Stevovic et al. (2001) reported that calcification and pre-seeding seed inoculation increased the alfalfa green matter outstandingly. In addition, it was found that microbiological fertilizers significantly increase the yield, primarily in the variants with calcification performed.

Medium SDW increase was noted with NPK fertilization and 15 t/ha of dolomite application, higher than in the treatment with inoculation and 15 t/ha of dolomite application. Fertilization without lime application (NØ) showed the same SDW as inoculation alone.

Table 2. Shoot parameters of alfalfa grown in acid soil

Treatment	Plant height (cm)	Shoot dry weight (mg/plant)	N% in SDW	N content in SDW (µg/plant)	Soil pH_{KCl}	Total soil N%
Control Ø	21.00^{c}	0.072^{d}	5.315^{a}	3.827^{e}	4.40^{e}	0.134^{a}
E.medicae	25.32^{bc}	0.109^{c}	4.451^{b}	4.317^{de}	4.36^{e}	0.131^{a}
E.medicae+E.meliloti	22.00^{c}	0.115^{c}	4.138^{cd}	4.759^{d}	4.34^{e}	0.117^{a}
E.meliloti	22.00^{c}	0.111^{c}	4.457^{b}	4.947^{d}	4.39^{e}	0.128^{a}
7$CaCO_3$+ *E.medicae+E.meliloti*	26.50^{bc}	0.113^{c}	4.296^{b}	4.854^{d}	6.21^{d}	0.123^{a}
15$CaCO_3$+ *E.medicae+E.meliloti*	31.68^{a}	0.230^{a}	3.731^{d}	8.581^{a}	7.20^{a}	0.121^{a}
15$CaMgCO_3$+ *E.medicae+E.meliloti*	24.00^{bc}	0.140^{bc}	4.214^{cd}	5.900^{c}	7.06^{b}	0.124^{a}
NØ+15$CaMgCO_3$	27.00^{ab}	0.180^{b}	4.008^{c}	7.214^{a}	7.14^{ab}	0.121^{a}
NØ	24.38^{bc}	0.116^{c}	4.448^{b}	5.160^{b}	4.21^{e}	0.132^{a}

Ø-control without inoculation, no fertilisation and no lime application; NØ-NPK fertilisation; 7$CaCO_3$-7 t/ha of lime; 15$CaCO_3$-15 t/ha of lime; 15$CaMgCO_3$-15 t/ha of dolomite; a-e: Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Table 3. Root parameters of alfalfa grown in acid soil

Treatment	Root length (cm)	Root dry weight (mg/plant)	N% in RDW	N content in RDW (µg/plant)
Control Ø	16.84^{bc}	0.029^{d}	3.356^{a}	0.973^{d}
E.medicae	19.00^{a}	0.055^{c}	2.726^{cd}	1.499^{b}
E.medicae+E.meliloti	18.00^{ab}	0.053^{c}	3.076^{b}	1.630^{b}
E.meliloti	15.06^{c}	0.050^{c}	3.071^{b}	1.536^{b}
7$CaCO_3$+ *E.medicae+E.meliloti*	17.33^{b}	0.055^{c}	2.814^{c}	1.548^{b}
15$CaCO_3$+ *E.medicae+E.meliloti*	17.18^{b}	0.090^{a}	2.667	2.400^{a}
15$CaMgCO_3$+ *E.medicae+E.meliloti*	15.50^{c}	0.045^{cd}	3.015^{b}	1.357^{bc}
NØ+15$CaMgCO_3$	15.00^{c}	0.071^{b}	2.494^{d}	1.771^{b}
NØ	16.00^{bc}	0.032^{d}	3.122^{ab}	0.999^{d}

Ø-control without inoculation, no fertilisation, no lime application; NØ-NPK fertilisation; 7$CaCO_3$-7 t/ha of lime; 15$CaCO_3$-15 t/ha of lime; 15$CaMgCO_3$-15 t/ha of dolomite; a-d: Means in a column followed by the same letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

It can be concluded that inoculation has the same effect as mineral fertilization if no lime materials are added. No increase in SDW was observed with 7 t/ha of lime, while outstanding increase was realized after 15 t/ha of lime application.

In the research of Katic et al. (2009) application of mineral fertilizers in alfalfa cultivation has no effect on the agrochemical properties of pseudogleys, but the addition of lime leads to a significant increase in alfalfa yield.

The maximum N% in shoot was obtained in the control treatment (5.32%) which had the lowest SDW, while the lowest N% was noted in the treatment with inoculation and 15 t/ha of lime (3.73%) which realized the biggest SDW. Except in the control where N% was high (>5%) in all other treatments it was optimal (2.5-4.9%). The SDW was in significant negative correlation with N% in shoot as well as with N% in root (Table 4). Nitrogen percentages in soils of different treatments correlated positively with N% in SDW. Increased plant growth and high dry matter production (2 fold higher over control) caused the dilution effect of N and its percentages decrease in plants of the treatments with higher SDW (Jarrell and Beverly, 1981; Timmer, 1991). Negative correlation of N% and SDW detected here was also established for some other nutrients in previous studies (Timmer, 1991; Imo, 2012). However, regardless of the N% decrease in inoculated and limed treatments, the total nitrogen content in plants (µg/plant) was increased significantly. In addition, SDW significantly correlated with total N content in SDW, plant height, as well as with soil pH and root dry weight (RDW).

In general, root dry weight (RDW) followed SDW, and RDW increased in the treatments with lime application and inoculation (Table 3). However, in contrast to SDW, RDW did not correlate positively with soil pH nor with root length.

Table 4. Correlations between alfalfa growth parameters

	Plant heigh t	SDW	N% in SDW	N content in SDW	Root length	RDW	N% in RDW	N in RDW	Soil pH	N% in soil
Plant height	1									
SDW	.852**	1								
N% in SDW	-.727*	-.835**	1							
N in SDW	.835**	.995**	-.793*	1						
Root length	.098	-.233	.054	-.307	1					
RDW	.842**	.867**	-.818**	.830**	.093	1				
N% in RDW	-.797*	-.690*	.743*	-.668*	-.050	-.834**	1			
N in RDW	.780*	.841**	-.815**	.796*	.122	.982**	-.729*	1		
Soil pH KCl	.710*	.786*	-.630	.799**	-.327	.659	-.645	.601	1	
N% in soil	-.388	-.601	.792*	-.549	-.001	-.664	.491	-.704*	-.559	1

*Correlation is significant at the 0.05 level, **Correlation is significant at the 0.01 level.

In agreement with previous studies we confirmed that it is possible to grow alfalfa on pseudogley soils, but the application of lime is necessary (Milic et al., 2014). Inoculation with acid tolerant strains improved alfalfa growth up to the same level as mineral fertilization without lime application. Inoculation with *E. medicae* strains did not have superior effect over inoculation with *E. meliloti* strains as it was expected. This is probably caused by similar pH tolerance of both strains regardless of their belonging to different species. Inoculations with rhizobia were found to significantly increase the yield, primarily in the variants with calcification performed, which is also in agreement with previous data.

CONCLUSIONS

Inoculation of alfalfa with *Ensifer* acid tolerant strains increased plant growth in very acid soil, but lime application together with inoculation provided the biggest shoot dry weight. Mineral fertilization without lime application improved alfalfa growth up to the same level as inoculation alone. To achieve proper alfalfa growth under acid soil conditions, rhizobial inoculation with the lime application is highly recommended.

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