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# Impact of Rhizobial and Mycorrhizal Inoculation on Alfalfa Cultivars Productivity under Different Liming Levels

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## ABSTRACT

**Alfalfa (*Medicago sativa* L.) is a highly valuable crop in agriculture, particularly due to its high biomass yield, rich protein content, and excellent digestibility, making it essential for livestock farming. However, its sensitivity to acidic soils presents a significant challenge for establishing and cultivating alfalfa in degraded soils. The current study aim was to investigate how selected inoculants affect alfalfa production when applied to acidic soils with different levels of liming. In this study, the effects of pre-sowing seed inoculation treatments of rhizobia - *Sinorhizobium (Ensifer) meliloti* (R), mycorrhizal fungi (MF), co-inoculation of rhizobia and mycorrhizal fungi (RMF), and control-no inoculation (C) -were assessed in three alfalfa cultivars (K-28, Zuzana, and Nijagara). The experiment was conducted under semi-controlled conditions in a completely randomized block design. The number of nodules, mycorrhizal colonization, dry matter yield of shoots and roots, protein yield, and phosphorus content were assessed. Acidic soil ($pH_{KCl}$ 4.55) was used for sowing, no lime (L0), alongside treatments on soils limed with 1 t $ha^{-1}$ (L1) and 2.5 t $ha^{-1}$ (L2) of $Ca(OH)_2$. The results indicated that rhizobia application increased the dry matter yield of shoots and roots. Protein yield at the L1 liming level ranged from 13.1 g/kg in the control treatment to 16.9 g/kg in the treatment with rhizobia, while at the L2 liming level, it ranged from 17.6 g/kg in the control treatment to 25.1 g/kg in the treatment with co-inoculation. Pre-sowing inoculation with mycorrhizal fungi and co-inoculation increased mycorrhizal colonization and phosphorus content. The results indicate the positive effects of both single and co-inoculation on alfalfa productivity. The impact of inoculation with the selected rhizobia strain was more pronounced in acidic soils than in limed soils and was cultivar-dependent. Therefore, the key to increasing alfalfa output in acidic soils is matching the strain with the cultivar.**

**Keywords:** *Medicago sativa*, AMF, nodulation, lime, yield.

## INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a crop with high biomass production, high protein, vitamin, and calcium content, and good digestibility, making it important for agriculture, especially in developed livestock production areas. However, its growth and persistence are reduced due to its sensitivity to increased soil acidity and $Al^{3+}$ ion toxicity (Khu et al., 2012, Bruma et al., 2023). The fertility of acidic soils is limited by high levels of $H^+$ and $Al^{3+}$ ions, other heavy metals, organic acids, nutrients deficiencies, and low microbial activity (Anđelković et al., 2010). One of the major advantages of alfalfa, like that of all legumes, is the ability to establish symbiotic nitrogen fixation, which is believed to have evolved around 58 million years ago and occurs in approximately 88% of legume species (De Faria et al., 1989). The most dominant genera with which alfalfa forms symbiosis are *Sinorhizobium meliloti*, *Sinorhizobium medicae*, and *Sinorhizobium tibeticum* (De Lajudie et al., 1994). Alfalfa can get 60-80% of its nitrogen needs from its partnership with rhizobia, but it's hard to create this partnership in acidic soils because the activity of rhizobia, as well as the formation and growth of nodules, is lower there. The symbiotic relationship between alfalfa and rhizobia coexists with its relationship with arbuscular mycorrhizal fungi (AMF). AMF represents the oldest and most widespread plant-fungal association worldwide (Bonfante and Genre, 2008). AMF play an important role in acidic soils because it helps plants to absorb more nutrients, especially phosphorus,

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by increasing the area of their roots, and it also helps plants to resist different stress factors. The hyphal networks of arbuscular fungi can directly bind $Al^{3+}$ ions, and they excrete organic (carboxylic) acids around the roots, which help transform to inactivate $Al^{3+}$ ions. This process constitutes the principal method for plants to overcome acid stress in the soil. The agricultural use of AMF and rhizobia has become widespread due to their ability to improve yield, reduce the need for fertilizer, and enhance plant health and disease resistance (Wu et al., 2022). The combined application of AMF and rhizobia increase nodule formation and enhances nitrogen fixation efficiency (Mohammadi et al., 2011). The application of liming as an agrotechnical measure alongside the use of tolerant genotypes and modern plant breeding techniques offers potential solutions to acidic soils. However, these methods may be costly or time-consuming. The development of biological agricultural systems is gaining particular importance in this regard, as adequate mycorrhization represents one of the mechanisms by which plants mitigate stress caused by soil acidity. Therefore, the use of biofertilizers offers both an economic and ecological alternative (Ben-Laouane et al., 2020). The role of co-inoculation with rhizobia and AMF, and their interaction in addressing the global challenge of soil acidity, which is a major limiting factor in alfalfa cultivation and, consequently, in the development of modern livestock farming, has not been fully researched in many parts of the world, including our region. This study aimed to research the effects of pre-sowing inoculation with *Sinorhizobium (Ensifer) meliloti* strain 218, an arbuscular mycorrhizal fungus, and their combined application on the dry matter yield and quality of three alfalfa cultivars on different liming levels.

## MATERIAL AND METHODS

### Experimental Design

The experiment was conducted in 2024 in a completely randomized block design with two factors. The first factor was different types of pre-sowing inoculations of rhizobia (R), arbuscular mycorrhizal fungi (MF), co-inoculation with rhizobia and arbuscular mycorrhizal fungi (RMF), and control with no inoculation (C). The second factor was three alfalfa cultivars. The alfalfa cultivars tested in the trial were K-28, which was created at the Institute for Forage Crops in Kruševac; Zuzana was obtained from the Research Institute for Fodder Crops in Troubsko, Czech Republic; and Nijagara was developed at the Institute of Field and Vegetable Crops in Novi Sad. A rhizobium strain, *Sinorhizobium (Ensifer) meliloti* 218, was obtained from the collection at the Institute of Soil Science, Belgrade, Serbia. The bacterial solution contained approximately $10^9$ cells/ml. As a source of AMF, commercial product Coveron® (Hello Nature International Srl, Italy) was used for the experiments. The commercial product contained *Rhizophagus intraradices* (300 spores/g) and *Funneliformis mosseae* (200 spores/g). Seeds of alfalfa varieties were sown in pots (20 pots per treatment). Pots were filled with approximately 1.5 kg of soil collected from a 0-30 cm depth that had undergone liming in the previous year using dehydrated calcium hydroxide $CaOH_2$ (>70% Ca). The soil had an acidic reaction with a pH of 4.55, an exchangeable acidity of 2.28 m.e. per 100 grams of soil, 9.45 mg of mobile aluminium per 100 grams of soil, a high total nitrogen content of 0.25%, medium levels of phosphorus at 11.06 mg per 100 grams of soil, medium potassium at 13.28 mg per 100 grams of soil, medium humus content at 2.83%, and low carbonate content at 1.4% CaO. Lime was applied to the soil surface in three levels: no lime (L0), 1 t $ha^{-1}$ (L1), and 2.5 t $ha^{-1}$ (L2), and immediately ploughed in the fall of 2023. Before sowing, 2 g of the commercial product Coveron® was added to the seeding site as MF treatment. The 2 ml bacterium solution of rhizobia was applied into pots as the R treatment. The same amounts of rhizobia and AMF inoculants were added in the co-inoculation treatments. To the control treatments, no inoculants were added. The pots were placed on tables in the greenhouse and watered as needed to maintain a relative soil moisture

content between 50% and 75% of field capacity.

**Measurements**

At the budding stage (approximately 70 days after emergence), the plants were analysed. The plants were harvested and green mass was measured. The alfalfa roots were carefully washed and weight. Nodule count was determined by counting nodules on alfalfa roots from all treatments. The root segment, approximately 0.2 g, was cleared with 10% KOH at 90°C, followed by rinsing and suspending in lactic acid for 7-10 minutes at room temperature. The roots were then stained with 0.05% trypan blue at 90°C for 20 minutes, according to the method of Phillips and Hayman (1970). The mycorrhizal colonisation was determined using the technique described by (Trouvelot et al., 1986). Random root fragments (1 cm in length) were placed on microscope slides in three replications, with ten root fragments per slide. Microscopic observation quantified the percentage of root mycorrhization, calculated as follows:

$$\% \text{ mycorrhization} = (N - N0) / N \times 100$$

where: N = number of observed root fragments and N0 = number of non-mycorrhized fragments.

The shoots and roots were dried at 80°C for 48 h and weighed to determine their dry matter. Dried alfalfa shoots were ground and sieved through a 0.5 mm sieve. The crude protein content was indirectly determined by measuring total nitrogen and multiplying by a factor of 6.25, following a modification of the Bremner method. Protein yield was calculated by multiplying the crude protein content by plant yield. Phosphorus content was determined using a spectrometric method (Animal feeding stuffs. Determination of phosphorus content. The spectrometric method used is outlined in JUS ISO 6491, published by the Federal Institute for Standardisation in 2002).

**Data Analysis**

The collected data were analysed using a two-factor ANOVA to see how pre-sowing inoculation, three types of alfalfa, and their interactions affected the measured parameters. Fisher's LSD test was used to compare treatment means at significance level of $P<0.05$. Principial component analysis (PCA) was used on the matrix of mean values for the studied parameters, and the axes with eigenvalues greater than 1 were graphically represented. All statistical analyses were done using STATISTICA 12 software (StatSoft, Inc., Tulsa, OK, USA).

## RESULTS AND DISCUSSION

**Nodule Number and Mycorrhizal Colonization**

The number of nodules per plant varied significantly depending on the application of inoculants and the level of liming. The lowest number of nodules, 120, was recorded in treatments with MF at the L1 level, whereas the highest number of nodules, 260, was observed in the treatment with R at the L0 level (Table 1).

*Table 1*. Number of nodules (#/plant) and mycorrhizal colonization (%) depending on pre-sowing inoculation of alfalfa varieties at different levels of liming (mean values ± standard error)

| | Number of nodules (#/plants) | | | Mycorrhizal Colonization (%) | | |
|---|---|---|---|---|---|---|
| LIME / INOCULUM | L0 | L1 | L2 | L0 | L1 | L2 |
| C | 188±10.2$^{b}$ | 194±4.78$^{b}$ | 253±7.45$^{a}$ | 45.0±1.67$^{b}$ | 35.0±2.52$^{c}$ | 30.6 ±2.66$^{c}$ |
| R | 260±7.73$^{a}$ | 254±9.28$^{a}$ | 223±5.05$^{b}$ | 41.8±1.42$^{b}$ | 43.7±2.35$^{b}$ | 35.7±2.31$^{bc}$ |
| RMF | 194±5.08$^{b}$ | 201±5.35$^{b}$ | 193±7.05$^{c}$ | 55.7±2.27$^{a}$ | 49.7±2.78$^{ab}$ | 43.7±2.35$^{a}$ |
| MF | 158 ±8.84$^{c}$ | 120±7.04$^{c}$ | 175±4.66$^{d}$ | 54.2±1.51$^{a}$ | 51.5±1.54$^{a}$ | 39.0±2.45$^{ab}$ |
| VARIETY | | | | | | |
| K-28 | 182±8.55$^{b}$ | 188±13.3$^{b}$ | 186± 7.11$^{c}$ | 50.0±2.44 | 48.5±2.48 | 37.6±1.68 |
| Nijagara | 186±11.1$^{b}$ | 174±9.96$^{c}$ | 227± 9.48$^{a}$ | 47.9±1.52 | 41.4±2.71 | 35.7±3.30 |
| Zuzana | 234±9.28$^{a}$ | 210±12.2$^{a}$ | 218± 6.05$^{b}$ | 49.5±2.78 | 44.9±2.75 | 38.5±2.29 |
| ANOVA | | | | | | |
| Inoculum | * | * | * | * | * | * |
| Variety | * | * | * | ns | ns | ns |
| Inoculum x Variety | * | * | * | ns | ns | ns |

*F-test is significant at the (P<0.05) level; ns - F-test is not significant; C - control; R - rhizobia; RMF - co-inoculation with rhizobia and mycorrhizal fungi; MF - mycorrhizal fungi. Different levels of liming: L0 - no lime; L1 - 1 t ha$^{-1}$; L2 - 2.5 t ha$^{-1}$. Values marked with different lowercase letters within columns differ significantly at the (P<0.05) level according to the LSD test.

Treatments with R showed significantly higher nodule numbers at L0 and L1 lime levels compared to other treatments, indicating the high efficacy of this rhizobia strain on acidic soil conditions. The use of efficient and more tolerant strains represents an alternative approach and way to increase nodulation and productivity of alfalfa on soils with lower pH values (Stević et al., 2010a). Numerous authors have reported the effectiveness of different rhizobia strains in establishing alfalfa nodulation under stressful conditions (Charman et al., 2008; Wigley et al., 2018). At the L2 level of liming, control treatments exhibited a higher number of nodules than those with rhizobia, suggesting possible competition between inoculated and native strains under conditions with higher calcium availability. The selected rhizobia strain for acidic soils may not ehance nodulation at higher lime levels due to competition with indigenous strains, which nodulate alfalfa plants much better in neutral soils than in acidic ones. Similar results were reported by Kopman et al. (1995), who found that inoculation with a selected strain and liming do not have positive effects on nodulation, which the authors explain by the low efficiency of the strain with the cultivar. Selected rhizobia inoculants must be highly effective in establishing nodulation and providing nitrogen-fixing plants ($N_2$-effectiveness). Native rhizobia strains often exhibit high competitiveness but low $N_2$-efficiency, thus, applied strains must be more competitive for nodulation (Checcucci et al., 2017; Onishchuk et al., 2017). At L0 and L1 liming levels, the variety Zuzana formed significantly more nodules than the other two varieties, while the variety K-28 had more nodules than Nijagara at the L1 liming level. The Nijagara variety showed significantly more number of nodules at the L2 liming level compared to the other two (Table 1). These results are consistent with the findings of Charman et al. (2008), which confirm that different alfalfa varieties form different numbers of nodules, suggesting a possible strain-specific response to the variety. The selection of appropriate rhizobia strains for pre-sowing inoculation is of significant importance because strains have different effects depending on soil response.

The number of nodules in different pre-sowing inoculation treatments for the three alfalfa varieties varied across different liming levels (Figure 1). Previous studies showed that different alfalfa varieties could display

different nodulation responses to the same rhizobia strain (Kang, 2019). The significantly higher nodules count at L0 and L1 in the Zuzana cultivar inoculated with the selected strain indicates a positive cultivar-strain interaction (Figure 1A and 1B). Similar cultivar-strain interactions have been reported by Kopman et al. (1995), and Han et al. (2024).

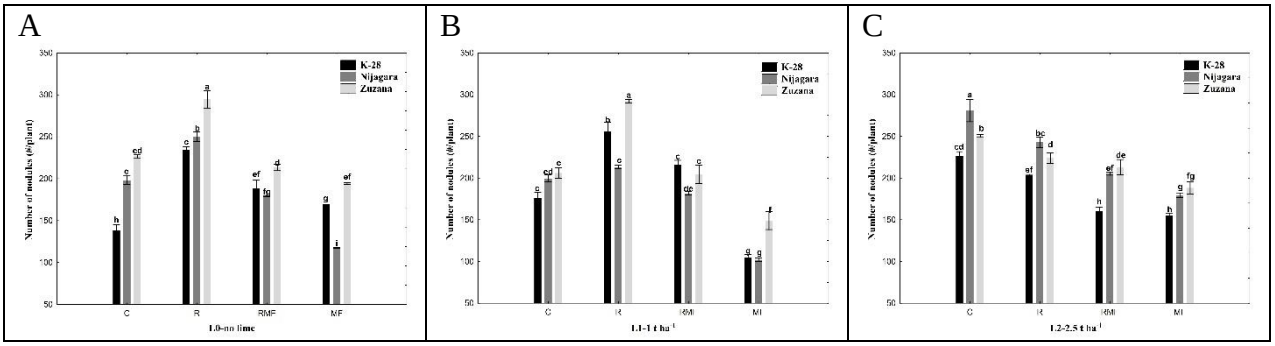


*Figure 1*. Effect of pre-sowing inoculation (C - control, R - rhizobia, RMF - co-inoculation of rhizobia and mycorrhizal fungi, MF - mycorrhizal fungi) and the different alfalfa varieties (K-28, Nijagara, and Zuzana) on number of nodules (#/plant) at different levels of liming: L0 - no lime (A), L1 - 1 t $ha^{-1}$ (B), and L2 - 2.5 t $ha^{-1}$ (C). Values marked with different lowercase letters on the graphs are significantly different at the (P< .05) level according to the LSD test.

At the L0 and L1 levels, the Zuzana variety showed the highest number of nodule in all inoculation treatments compared to the other varieties. At the L2 level of liming, the Nijagara variety had the highest number of nodules in C and R treatments compared to the other treatments and varieties (Figure 1C). Variations in nitrogen fixation efficiency among *Sinorhizobium (Ensifer) meliloti spp.* strains, ranging from low to high, were influenced by alfalfa variety and the year of use (Delić et al., 2013).

Pre-sowing inoculants significantly influenced mycorrhizal colonization at all three liming levels, although no differences were noted among the alfalfa varieties. The MF and RMF treatments showed significantly higher mycorrhizal colonization compared to C treatments at all liming levels (Table 1). Both single mycorrhizal inoculation and co-inoculation of rhizobia and mycorrhizal fungi enhanced mycorrhizal colonization in alfalfa plants (Ashrafi et al., 2014).

**The dry matter yield of shoots and roots**

The dry matter yield of shoots and roots pre-sowing inoculation with the rhizobia strain had a significant impact on the dry matter yield of shoots at the L0 and L1 levels of liming, demonstrating the strain's effectiveness in improving dry matter yield, praticulary under low calcium and high acidity conditions. However, at the L2 liming level, there were no significant yield differences between the R and RMF treatments (Table 2). Our results confirm the positive effects of this strain, as they align with two years of research on slightly acidic soil of the eutric cambisol type ($pH_{KCl}$ 6.22), where Buntić et al. (2021) observed an increase in the dry matter yield of alfalfa shoots with the use of the liquid rhizobia inoculant *Sinorhizobium (Ensifer) meliloti* strain 218 compared to the control. Positive effects of pre-sowing inoculation with a pH-resistant rhizobia strain on significantly increased dry matter yield in all alfalfa varieties during the first two years of the experiment are also supported by research (Stević et al., 2010a).

Co-inoculation treatments with rhizobia and mycorrhizal fungi showed a significant increase in the dry matter yield of shoots compared to the MF and C treatments at all liming levels, which is also consistent with the research by Ben-Laouane et al. (2020**)** demonstrating that the symbiosis between rhizobia and fungi can improve nutrient uptake and increase yield under stressful

conditions. The MF treatment also achieved significantly greater dry matter yield effects compared to C at all liming levels, highlighting the importance of pre-sowing inoculation with fungi to improve alfalfa productivity, especially on acidic soils. At the L0 liming level, the K-28 variety produced a significantly higher dry matter yield than the other two varieties (Table 2).

*Table 2*. The dry matter yield of shoots and roots (g/per plant) depending on pre-sowing inoculation of alfalfa varieties at different liming levels (mean values ± standard error)

| | Dry matter of shoots (g/DM) | | | Dry matter of roots (g/DM) | | |
|---|---|---|---|---|---|---|
| LIME / INOCULUM | L0 | L1 | L2 | L0 | L1 | L2 |
| C | 0.84±0.01$^{d}$ | 1.13±0.04$^{d}$ | 1.09±0.02$^{c}$ | 0.91±0.02$^{b}$ | 0.90±0.01$^{b}$ | 0.98±0.01$^{c}$ |
| R | 1.06±0.02$^{a}$ | 1.40±0.03$^{a}$ | 1.38 ±0.02$^{a}$ | 1.17±0.03$^{a}$ | 0.95±0.02$^{a}$ | 1.13±0.02$^{a}$ |
| RMF | 0.90±0.01$^{b}$ | 1.28±0.03$^{b}$ | 1.37 ±0.03$^{a}$ | 0.72±0.01$^{c}$ | 0.79±0.03$^{d}$ | 1.10±0.02$^{b}$ |
| MF | 0.87±0.01$^{c}$ | 1.24±0.02$^{c}$ | 1.23 ±0.01$^{b}$ | 0.67±0.01$^{d}$ | 0.86±0.03$^{c}$ | 1.13±0.02$^{a}$ |
| VARIETY | | | | | | |
| K-28 | 0.94±0.03$^{a}$ | 1.25±0.03$^{b}$ | 1.25±0.01$^{b}$ | 0.84±0.04$^{c}$ | 0.87±0.01$^{b}$ | 1.08±0.01$^{b}$ |
| Nijagara | 0.91±0.02$^{b}$ | 1.16±0.03$^{c}$ | 1.27±0.04$^{a}$ | 0.91±0.06$^{a}$ | 0.82±0.02$^{c}$ | 1.07±0.02$^{b}$ |
| Zuzana | 0.90±0.02$^{b}$ | 1.37±0.03$^{a}$ | 1.29±0.04$^{a}$ | 0.86±0.04$^{b}$ | 0.94±0.03$^{a}$ | 1.11±0.02$^{a}$ |
| ANOVA | | | | | | |
| Inoculum | * | * | * | * | * | * |
| Variety | * | * | * | * | * | * |
| Inoculum x Variety | * | * | * | * | * | * |

*F-test is significant at the (P<0.05) level; ns - F-test is not significant; C - control; R - rhizobia; RMF - co-inoculation with rhizobia and mycorrhizal fungi; MF - mycorrhizal fungi. Different levels of liming: L0 - no lime; L1 - 1 t ha$^{-1}$; L2 - 2.5 t ha$^{-1}$. Values marked with different lowercase letters within columns differ significantly at the (P<0.05) level according to the LSD test.

Stević et al. (2010b) evaluated the acidity tolerance of five alfalfa varieties (Banat, Medijana, K-28, Sinskaya, and OS-66). The K-28 variety had a significantly higher dry matter yield than the other varieties, which indicates its tolerance for lower pH values and confirms the results of our research. Treatments R and RMF with alfalfa varieties at all liming levels led to a substantial increase in dry matter yield of shoots in comparison to the MF and C treatments (Figure 2). Larimer et al. (2014) highlight the strong synergistic effect of co-inoculation with rhizobia and mycorrhizal fungi, independent of nutrient effects, on biomass production in legumes in their research.

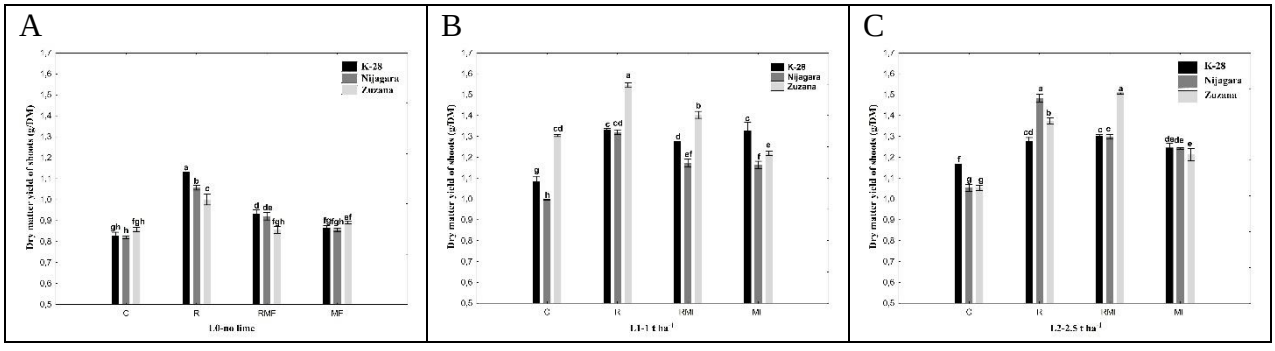


*Figure 2*. Effect of pre-sowing inoculation (C - control, R - rhizobia, RMF - co-inoculation of rhizobia and mycorrhizal fungi, MF - mycorrhizal fungi) and the different alfalfa varieties (K-28, Nijagara, and Zuzana) on dry matter yield of shoots (g/DM) at different levels of liming: L0 - no lime (A), L1 - 1 t ha$^{-1}$ (B), and L2 - 2.5 t ha$^{-1}$ (C). Values marked with different lowercase letters on the graphs are significantly different at the P<0.05 level according to the LSD test.

Delić et al. (2013) found that rhizobia strains, host varieties, and interactions between them have a significant impact on the dry matter yield of alfalfa shoots. This analysis is consistent with our findings, where the R treatments with the K-28 variety at the L0 level (Figure 2A), Zuzana at the L1 level (Figure 2B), and Nijagara at the L2 level (Figure 2C) achieved the best results. The study by Stević et al. (2007) also observed that more tolerant strains improve soil acidity and dry matter yield in different alfalfa varieties. The synergistic effect of rhizobia and mycorrhizal fungi improves plant yield (Guo et al., 2010; Xie et al., 2020). RMF treatments at the L1 and L2 liming levels in the Zuzana variety lead to significantly higher dry matter levels compared to other treatments, which is consistent with reports by previous authors.

The R treatment had the highest significant dry matter yield of roots compared to the other treatments and the control at the L0 and L1 liming levels (Table 2). According to Stajković-Srbinović et al. (2015), pre-sowing inoculation with selected and refined *Sinorhizobium (Ensifer) meliloti* strains positively affected the dry matter yield of roots in very acidic soil ($pH_{KCl}$ 4.4). Single inoculation with AMF and co-inoculation with rhizobia at liming levels L0 and L1 resulted in significantly lower dry matter yields for roots compared to uninoculated plants. This may be because mycorrhizal fungi increase the efficiency of nutrient and water absorption through their hyphal networks, leading to a reduced need for the development of a large root system (Rillig et al., 2002; Johnson et al., 2010; Smith and Read, 2010). At the L2 liming level, all pre-sowing inoculation treatments resulted in significantly higher dry matter yield of roots in alfalfa compared to the control. The Nijagara variety at the L0 liming level had a significantly higher dry matter yield of roots compared to K-28 and Zuzana, while Zuzana had substantially higher values than K-28. It is consistent with the findings of Grewal and Williams (2003), who note that different varieties significantly differ in root growth at certain pH values. At the L1 and L2 liming levels, the Zuzana variety had a significantly higher dry matter yield of roots compared to the other two varieties, suggesting that this variety has a greater potential for root growth in conditions with higher calcium levels (Table 2).

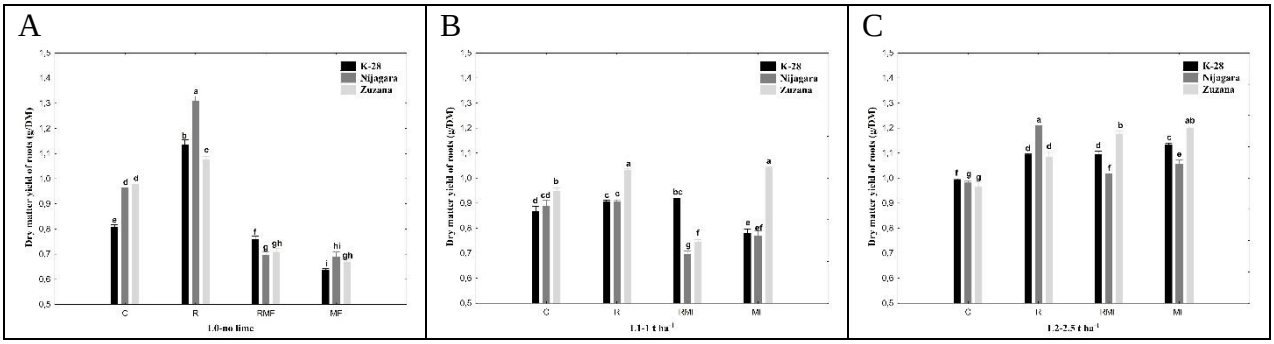


*Figure 3*. Effect of pre-sowing inoculation (C - control, R - rhizobia, RMF - co-inoculation of rhizobia and mycorrhizal fungi, MF - mycorrhizal fungi) and the different alfalfa varieties (K-28, Nijagara, and Zuzana) on dry matter yield of roots (g/DM) at different levels of liming: L0 - no lime (A), L1 - 1 t $ha^{-1}$ (B), and L2 - 2.5 t $ha^{-1}$ (C). Values marked with different lowercase letters on the graphs are significantly different at the ($P<0.05$) level according to the LSD test.

Single inoculation with mycorrhizal fungi and co-inoculation with rhizobia and mycorrhizal fungi, with all varieties, resulted in significantly lower yields compared to non-inoculated treatments at the L0 liming level (Figure 3A). The lower dry matter yield of roots in different varieties in these treatments and liming levels is attributed to a combination of factors. These include low pH values that inhibit root growth (Berenji et al., 2017), as well as the fact that arbuscular mycorrhizal fungi can modify root

architecture and the morphology of their host plants (Ren et al., 2016), where AMF establish greater symbiosis and take over the role of nutrient and water absorption (Johnson et al., 2010). The Zuzana variety at the L1 liming level in the R and MF treatments showed a significantly increased dry matter yield of roots compared to other varieties and treatments (Figure 3B). Nijagara in the R treatments and Zuzana in the MF and RMF treatments recorded significantly higher dry matter yield of roots at the L2 liming level (Figure 3C).

**Protein Yield and P Content**

The protein yield per alfalfa plant varied at different liming levels. In the soil without lime, protein yield ranged from 13.1 g/kg in the control treatment to 16.9 g/kg in the R treatment, while in the soil treatment with 2.5 t $ha^{-1}$ lime, it ranged from 17.6 to 25.1 g/kg (Table 3).

*Table 3*. Protein yield (g/kg DM) and P content (mg/g DM) depending on pre-sowing inoculation of alfalfa varieties at different liming levels (mean values ± standard error)

| | Protein yield (g/kg plant) | | | P content (mg/g plant) | | |
|---|---|---|---|---|---|---|
| LIME / INOCULUM | L0 | L1 | L2 | L0 | L1 | L2 |
| C | 13.1±0.46$^{d}$ | 21.0±0.36$^{c}$ | 17.6±0.43$^{d}$ | 3.43±0.15$^{c}$ | 3.68±0.09$^{c}$ | 4.16±0.01$^{c}$ |
| R | 16.9±0.46$^{a}$ | 26.2±0.29$^{a}$ | 24.2±0.69$^{b}$ | 4.01±0.17$^{b}$ | 4.20±0.06$^{b}$ | 4.14±0.01$^{c}$ |
| RMF | 15.7±0.46$^{b}$ | 22.2±0.37$^{b}$ | 25.1±0.36$^{a}$ | 4.28±0.14$^{b}$ | 4.68±0.13$^{a}$ | 4.89±0.01$^{b}$ |
| MF | 13.6±0.18$^{c}$ | 23.2±0.76$^{b}$ | 20.7±0.26$^{c}$ | 4.82±0.18$^{a}$ | 4.41±0.11$^{b}$ | 6.20±0.00$^{a}$ |
| VARIETY | | | | | | |
| K-28 | 16.3±0.53$^{a}$ | 23.2±0.45$^{ab}$ | 22.1±0.60$^{a}$ | 4.00±0.20 | 4.11±0.15$^{b}$ | 4.81±0.27 |
| Nijagara | 15.1±0.23$^{b}$ | 22.4±0.61$^{b}$ | 22.1±0.95$^{a}$ | 4.14±0.13 | 4.22±0.08$^{ab}$ | 4.82±0.26 |
| Zuzana | 13.2±0.39$^{c}$ | 23.8±0.68$^{a}$ | 21.4±0.74$^{b}$ | 4.24±0.25 | 4.40±0.16$^{a}$ | 4.93±0.26 |
| ANOVA | | | | | | |
| Inoculum | * | * | * | * | * | * |
| Variety | * | * | * | ns | * | ns |
| Inoculum x Variety | * | * | * | ns | * | ns |

*F-test is significant at the (P<0.05) level; ns - F-test is not significant; C - control; R - rhizobia; RMF - co-inoculation with rhizobia and mycorrhizal fungi; MF - mycorrhizal fungi. Different levels of liming: L0 - no lime; L1 - 1 t $ha^{-1}$; L2 - 2.5 t $ha^{-1}$. Values marked with different lowercase letters within columns differ significantly at the (P<0.05) level according to the LSD test.

Pre-sowing inoculation treatments with rhizobia led to a significant increase in protein yield in alfalfa plants compared to other treatments at the L0 and L1 liming levels. At the L2 level, the RMF treatment resulted in a significantly higher protein yield than the others. Positive effects on protein yield in plants with individual inoculation of rhizobia and co-inoculation with rhizobia and AMF have also been reported by Stajković-Srbinović et al. (2017) and Xie et al. (2020). The varieties exhibited different protein yields across at the liming levels (Table 3). The K-28 variety recorded the highest protein yield at the L0 and L2 levels, along with the Nijagara variety, while at the L1 level, Zuzana recorded the best results. Other authors have also reported different protein yields for various alfalfa varieties grown on acidic soils (Milić et al., 2014; Han et al., 2024). Protein yield under different pre-sowing inoculation treatments varied with alfalfa variety and liming level (Figure 4). At the L0 level, the K-28 variety in the R and RMF treatments recorded significantly higher protein yields compared to other varieties (Figure 4A). Similar findings have been reported by other authors who observed improved protein yield and alfalfa productivity when K-28 was inoculated with rhizobia strains more tolerant to low pH (Delić et al., 2010; Stajković-Srbinović et al., 2015).

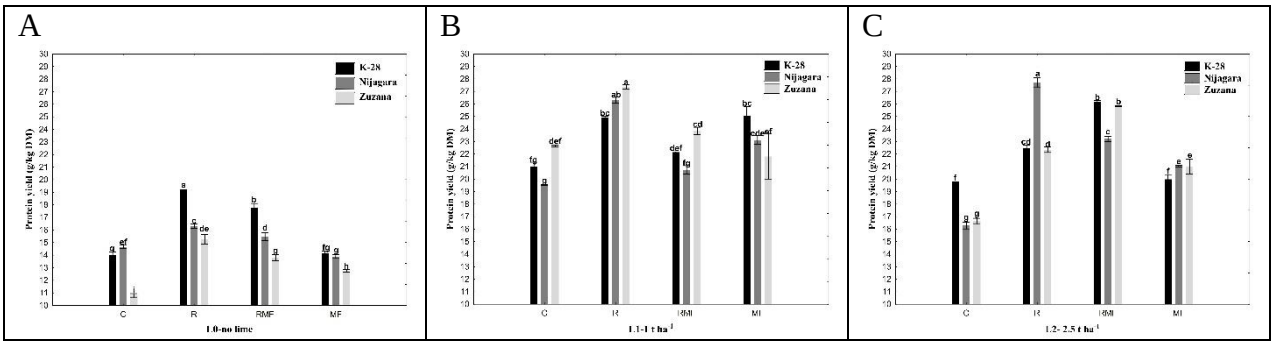


*Figure 4.* Effect of pre-sowing inoculation (C - control, R - rhizobia, RMF - co-inoculation of rhizobia and mycorrhizal fungi, MF - mycorrhizal fungi) and the different alfalfa varieties (K-28, Nijagara, and Zuzana) on protein yield (g/kg DM) at different levels of liming: L0 - no lime (A), L1 - 1 t ha$^{-1}$ (B), and L2 - 2.5 t ha$^{-1}$ (C). Values marked with different lowercase letters on the graphs are significantly different at the ($P<0.05$) level according to the LSD test.

At the L1 and L2 liming levels, all varieties generally produced higher protein yields per plant compared to the L0 level. At the L1 level, all three varieties in the R treatment, as well as K-28 in the MF treatment achieved higher protein yields per plant than other combinations (Figure 4B). At the L2 liming level, Nijagara in the R treatment, and K-28 and Zuzana in the RMF treatment, recorded significantly higher protein yields than the other varieties and treatments (Figure 4C). Positive effects of co-inoculation on nitrogen content and protein yield in alfalfa have also been confirmed by Xie et al. (2020) and Gatabazi et al. (2023). The MF and RMF treatments led to significantly higher phosphorus content in plants at all three levels compared to uninoculated plants (Table 3). The R treatment had a significantly effects only at the L0 and L1 liming levels, where increased phosphorus content compared to the controls. These results suggest that AMF inoculation, both alone and in combination with rhizobia, plays a crucial role in enhancing phosphorus uptake by host plants. Similar findings on improved phosphorus content in alfalfa sprouts under stress conditions with individual rhizobia inoculation and co-inoculation with AMF have been reported by Pereira et al. (2019) and Duan et al. (2024).

Among the varieties at the L1 level, significant differences in phosphorus content were observed, with Zuzana recording higher values than K-28. At the other liming levels, differences among varieties were not statistically significant (Table 3). These findings align with the research of Ashrafi et al. (2014), who reported that phosphorus content can be influenced by the variety, nothing variability among three alfalfa varieties.

Significant differences in phosphorus content at the L1 liming level were observed only in treatments involving pre-sowing inoculants (Figure 5). The highest phosphorus content was recorded in the Zuzana variety with the RMF treatment, which did not differ significantly from the Zuzana variety with MF treatment or from K-28 variety with the MF treatment. The beneficial effects of single and co-inoculations on phosphorus content in alfalfa and other leguminous species have been reported (Guo et al., 2010; Huang et al., 2017). In the control and R treatments, all varieties showed uniformly lower phosphorus content values.

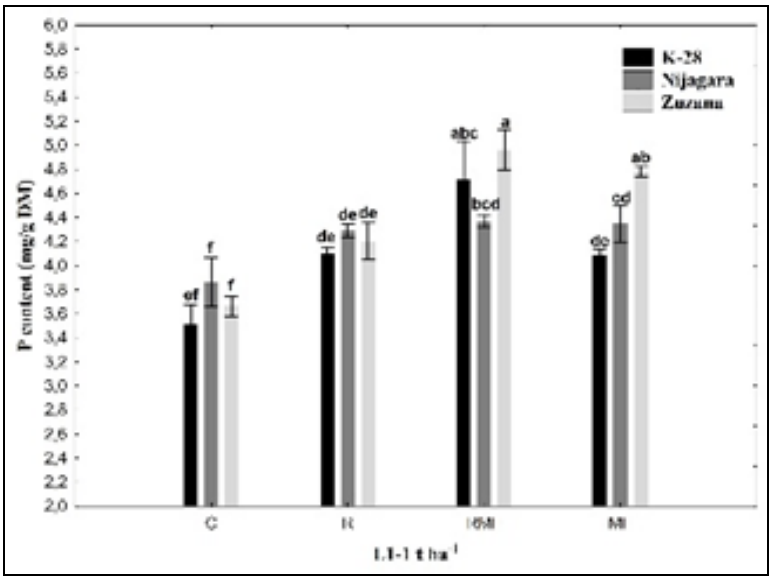


*Figure 5.* Effect of pre-sowing inoculation (C - control, R - rhizobia, RMF - co-inoculation of rhizobia and mycorrhizal fungi, MF - mycorrhizal fungi) and the different alfalfa varieties (K-28, Nijagara, and Zuzana) on P content (mg/g DM) at L1 - 1 t $ha^{-1}$ liming level. Values marked with different lowercase letters on the graphs differed significantly at the level of ($P<0.05$) according to the LSD test.

**PCA analysis**

The first two principial components variables explain 73.2% of the total variation. The first principal component, PCA1, explains 43.7%, while the second component, PCA2, explains 29.5% (Figure 6).

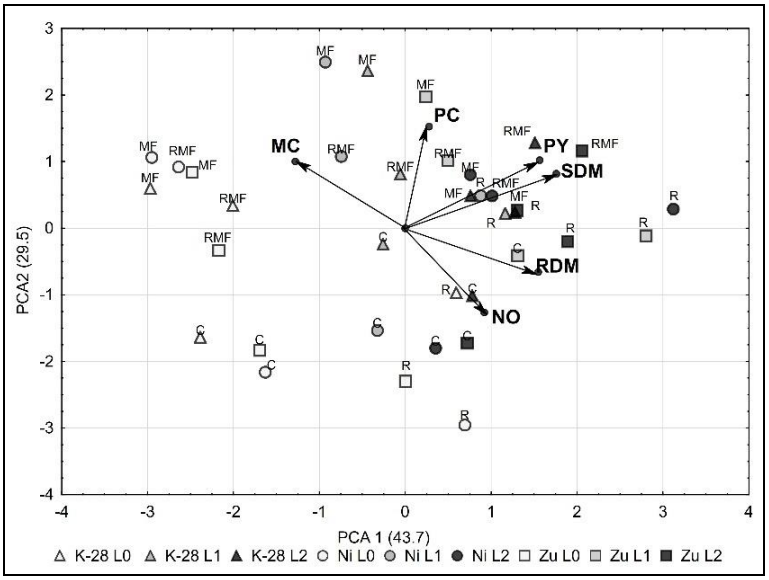


*Figure 6.* The biplot of the first two components of the principal component analysis (PCA) illustrates the differences influenced by inoculation (C - control, R - rhizobia, RMF - co-inoculation of rhizobia and mycorrhizal fungi, and MF - mycorrhizal fungi) and alfalfa varieties (K-28, Nijagara, and Zuzana) at different levels of liming (L0 - without lime, L1 - 1 t $ha^{-1}$, and L2 - 2.5 t $ha^{-1}$) on the number of nodules (NO), mycorrhizal colonisation (MC), dry matter yield of shoots (SDM), dry matter yield of roots (RDM), protein yield (PY), and phosphorus content (PC).

Treatments with mycorrhizal fungi and co-inoculation of rhizobia and mycorrhizal fungi at the L0 level of liming were grouped around the parameter mycorrhizal colonisation, while at the L1 level of liming, these treatments were grouped around the parameters phosphorus content, indicting a notable contribution to their increase. This is consistent with findings that have shown that AMF and co-inoculation with rhizobia increase mycorrhization and enhance nutrient absorption, especially phosphorus, in plants (Duan et al. 2024). The

close vector orientation the dry matter yield of shoots and protein yield indicate a strong positive correlation, with treatments R, MF, and RMF showing a significant contribution to increasing these parameters at the L1 and L2 levels of liming. Single inoculations with rhizobia, mycorrhizal fungi, and co-inoculation with rhizobia and fungi lead to increased productivity in various parameters in alfalfa (Guo et al., 2010; Ben-Laouane et al., 2020). On the other hand, the dry matter yield of roots and the number of nodules grouped around the R treatments suggest that treatments that increase mycorrhization and phosphorus uptake do not simultaneously affect root development and nodulation. Positioning the control treatment far from the coordinate origin indicates significant differences compared to treatments with rhizobia, mycorrhizal fungi, and their co-inoculations.

## CONCLUSIONS

Selecting appropriate strains of rhizobia and applying them in combination with mycorrhizal fungi can significantly improve alfalfa productivity in acidic soils, as demonstrated in this study. Rhizobia application led to a significant increase in the number of nodules and dry matter yield, with the most pronounced effects observed at lower levels of liming. In contrast, AMF played a key role in improving phosphorus uptake across all liming levels. Co-inoculation resulted in increased protein yield, dry matter yield of shoots, and phosphorus content, with the most substantial effect on protein yield at L2 liming levels. The alfalfa varieties studied responded differently to inoculant application depending on the liming level, highlighting the specificity of strains-variety interactions. Future research should focus on field trials to confirm the effectiveness of inoculation under natural agro-ecological conditions and assess its long-term effects on soil fertility and the sustainability of alfalfa production. Special attention should be given to the interaction between inoculants and various agronomic practices, such as liming and the application of biostimulants, to further optimise their use. Increasing alfalfa productivity on acidic soils also requires the selection of more tolerant alfalfa varieties and compatible strains of microorganisms. These and other strategies would contribute to the development of sustainable solutions for enhancing alfalfa productivity on acidic soils, while reducing reliance on mineral fertilizers and supporting ecological sustainability.

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## REFERENCES

Anđelković, S., Jarak, M., Radović, J., Vasić, T., Živković, B., Đurić, S., 2010. *The influence of different inoculants on the alfalfa grown on acid soil*. Animal Husbandry, 26: 611-617.

Ashrafi, E., Zahedi, M., Razmjoo, J., 2014. *Co-inoculations of arbuscular mycorrhizal fungi and rhizobia under salinity in alfalfa.* Soil Science and Plant Nutrition, 60(5): 619-629.

Ben-Laouane, R., Baslam, M., Ait-El-Mokhtar, M., Anli, M., Boutasknit, A., Ait-Rahou, Y., Toubali, S., Mitsui, T., Oufdou, K., Wahbi, S., Meddich, A., 2020. *Potential of Native Arbuscular Mycorrhizal Fungi, Rhizobia, and/or Green Compost as Alfalfa (Medicago sativa) Enhancers under Salinity.* Microorganisms, 8(11): 1695.

Berenji, S., Moot, D.J., Moir, J.L., Ridgway, H., Rafat, A., 2017. *Dry matter yield, root traits, and nodule occupancy of lucerne and Caucasian clover when grown in acidic soil with high aluminium concentrations.* Plant and Soil, 416: 227-241.

Bonfante, P., and Genre, A. 2008. *Plants and arbuscular mycorrhizal fungi: an evolutionary-developmental perspective.* Trends in Plant Science, 13(9): 492-498.

Bruma, I.S., Toader, M., Popescu, G., Petcu, V., Georgescu, E., 2023. *The evolution of alfalfa, as important crop in organic farming system in Romania*. Romanian Agricultural Research, 40: 297-306. https://doi.org/10.59665/rar4028

Buntić, A., Stajković-Srbinović, O., Knežević, M., Rasulić, N., Ugrenović, V., Kuzmanović, D., Delić, D., 2021. *Efficiency of alfalfa seed priming and rhizobial-based liquid inoculants in an Eutric Cambisol.* Zemdirbyste-Agriculture, 108(4): 339-346.

Charman, N., Ballard, R.A., Humphries, A.W., Auricht, G.C., 2008. *Improving lucerne nodulation at low pH: contribution of rhizobial and plant genotype to the nodulation of lucerne seedlings growing in solution culture at pH 5*. Australian Journal of Experimental Agriculture, 48(4): 512-517.

Checcucci, A., DiCenzo, G.C., Bazzicalupo, M., Mengoni, A., 2017. *Trade, diplomacy, and warfare: the quest for elite rhizobia inoculant strains.* Frontiers in Microbiology, 8: 2207.

De Lajudie, P., Willems, A., Pot, B., Dewettinck, D., Maestrojuan, G., Neyra, M., Collins, D.M., Dreyfus, B., Kersters, K., Gillis, M., 1994. *Polyphasic taxonomy of rhizobia: emendation of the genus Sinorhizobium and description of Sinorhizobium meliloti comb. nov., Sinorhizobium saheli sp. nov., and Sinorhizobium teranga sp. nov.* International Journal of Systematic Bacteriology, 44(4): 715-733.

De Faria, S.M., Lewis, G.P., Sprent, J.I., Sutherland, J.M., 1989. *Occurrence of nodulation in the Leguminosae.* New Phytologist, 111(4): 607-619.

Delić, D., Stajković, O., Radović, J., Stanojković, A., Kuzmanović, Đ., Rasulić, N., Miličić, B., 2010. *Genotypic Differences in Symbiotic N2 Fixation of Some Alfalfa (Medicago sativa L.) Genotypes. In Sustainable use of genetic diversity in forage and turf breeding*. Springer Netherlands: 79-84.

Delić, D., Stajković-Srbinović, O., Radović, J., Kuzmanović, D., Rasulić, N., Simić, A., Knežević-Vukčević, J., 2013. *Differences in symbiotic N2 fixation of alfalfa, Medicago sativa L. cultivars and Sinorhizobium spp. strains in field conditions.* Romanian Biotechnological Letters, 18(6): 8743-8750.

Duan, H.X., Luo, C.L., Wang, X., Cheng, Y.S., Abrar, M., Batool, A., 2024. *Responses of Legumes to Rhizobia and Arbuscular Mycorrhizal Fungi under Abiotic Stresses: A Global Meta-Analysis.* Agronomy, 14(11): 2597.

Gatabazi, A., Botha, M., Mvondo-She, M.A., 2023. *Assessing liquid inoculant formulation of biofertilizer (Sinorhizobium meliloti) on growth, yield, and nitrogen uptake of lucerne (Medicago sativa).* Nitrogen, 4(1): 125-134.

Grewal, H.S., and Williams, R., 2003. *Liming and cultivars affect root growth, nodulation, leaf to stem ratio, herbage yield, and elemental composition of alfalfa on an acid soil.* Journal of Plant Nutrition, 26(8): 1683-1696.

Guo, Y., Ni, Y., Huang, J., 2010. *Effects of rhizobium, arbuscular mycorrhiza and lime on nodulation, growth and nutrient uptake of lucerne in acid purplish soil in China.* Tropical Grasslands, 44(2): 109-114.

Han, Y., Kang, W., Shi, S., Guan, J., Du, Y., He, F., Lu, B., Wang, M., 2024. *Investigation of Nitrogen Fixation Efficiency in Diverse Alfalfa Varieties Utilizing Sinorhizobium meliloti LL2.* Agronomy, 14(11): 2732.

Huang, L., He, Y., Guo, Y., 2017. *The efficiency of arbuscular mycorrhizal fungi in promoting alfalfa growth in acid soils.* Journal of Agricultural Science, 9: 186-204.

Johnson, N.C., Wilson, G.W., Bowker, M.A., Wilson, J.A., Miller, R.M. 2010. *Resource limitation is a driver of local adaptation in mycorrhizal symbioses.* Proceedings of the National Academy of Sciences, 107(5): 2093-2098.

Kang, W.J., 2019. *Biotype Classification of Medicago Sativa Rhizobia and Its Transcriptome Analysis.* Ph.D. Thesis, Gansu Agricultural University, Lanzhou, China.

Khu, D.M., Reyno, R., Brummer, E.C., Monteros, M.J., 2012. *Screening methods for aluminum tolerance in alfalfa*. Crop Science, 52(1): 161-167.

Koopman, D.J., Tow, P.G., Reeves, T.G., Gibson, A.H., 1995. *Soil acidification, chlorsulfuron application and Rhizobium meliloti as factors in lucerne yield decline.* Soil Biology and Biochemistry, 27(4-5): 673-677.

Larimer, A.L., Clay, K., Bever, J.D., 2014. *Synergism and context dependency of interactions between arbuscular mycorrhizal fungi and rhizobia with a prairie legume.* Ecology, 95(4): 1045-1054.

Milić, D., Katić, S., Katanski, S., Dugalić, G., Bokan, N., Vasiljević, S., 2014. *Effect of genotype and applied management on alfalfa yield and quality.* Field and Vegetable Crops Research, 51(2): 91-99.

Mohammadi, K., Khalesro, S., Sohrabi, Y., Heidari, G., 2011. *A review: beneficial effects of the mycorrhizal fungi for plant growth.* Journal of Applied Environmental and Biological Sciences, 1(9): 310-319.

Onishchuk, O.P., Vorobyov, N.I., Provorov, N.A., 2017. *Nodulation competitiveness of nodule bacteria: genetic control and adaptive significance.* Applied Biochemistry and Microbiology, 53: 131-139.

Pereira, S., Mucha, Â., Gonçalves, B., Bacelar, E., Látr, A., Ferreira, H., Oliveira, I., Rosa, E., Marques, G., 2019. *Improvement of some growth and yield parameters of faba bean (Vicia faba) by inoculation with Rhizobium laguerreae and arbuscular mycorrhizal fungi.* Crop and Pasture Science, 70(7): 595-605.

Phillips, J.M., and Hayman, D.S., 1970. *Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection.* Transactions of the British Mycological Society, 55(1): 158-161, IN16-IN18.

Ren, A., Waly, N., Lu, W., Ma, C., Zhang, Q., Liu, H., Yang, J., 2016. *Mycorrhizal Glomus spp. vary in their effects on the dynamics and turnover of fine alfalfa (Medicago sativa L.) roots.* Pakistan Journal of Botany, 48(2): 793-798.

Rillig, M.C., Wright, S.F., Eviner, V.T., 2002. *The role of arbuscular mycorrhizal fungi and glomalin in soil aggregation: comparing effects of five plant species.* Plant and Soil, 238: 325-333.

Smith, S.E., and Read, D.J., 2010. *Mycorrhizal symbiosis.* Academic Press.

Stajković-Srbinović, O., Delić, D., Rasulić, N., Buntić, A., Kuzmanović, D., Kostić-Kravljanac, L., Sikirić, B., 2015. *Alfalfa growth on acid soil as influenced by liming and Ensifer strains inoculation.* Plant and Soil, 64(2): 1-8.

Stajković-Srbinović, O., Delić, D., Nerandžić, B., Andjelović, S., Sikirić, B., Kuzmanović, Dj., Rasulić, N., 2017. *Alfalfa yield and nutrient uptake as influenced by co-inoculation with rhizobium and rhizobacteria.* Romanian Biotechnological Letters, 22(4): 12834-12841.

Stevović, V., Đukić, D., Đurović, D., Mandić, L., 2007. *Effects of seed inoculation and liming on forage yield and quality of alfalfa and red clover.* Field and Vegetable Crops Research, 44(1): 253-26.

Stevović, V., Lazarević, B., Đurović, D., Beković, D., Tomić, D., 2010a. *Biomass yield and quality of alfalfa cultivars grown on pseudogley soil.* In: Marić, S., Lončarić, Z. (eds.), 45. Hrvatski i 5. Međunarodni Simpozij Agronoma, Opatija, Hrvatska, 15-19 veljače 2010: 930-934.

Stevović, V., Đurović, D., Đukić, D., Lazarević, B., Tomić, D., 2010b. *Alfalfa response to low soil pH and liming.* Biotechnology in Animal Husbandry, 26: 261-269.

Trouvelot, A., Kough, J., Gianinazzi-Pearson, V., 1986. *Mesure du taux de mycorhization VA d'un système radiculaire. Recherche de méthodes d'estimation ayant une signification fonctionnelle.* In: Gianinazzi, S., Gianinazzi-Pearson, V. (eds.) Physiological and Genetical Aspects of Mycorrhizae. INRA Press: 217-221.

Wigley, K., Ridgway, H.J., Humphries, A.W., Ballard, R.A., Moot, D.J., 2018. *Increased lucerne nodulation in acid soils with Sinorhizobium meliloti and lucerne tolerant to low pH and high aluminium.* Crop and Pasture Science, 69(10): 1031-1040.

Wu, Y., Li, Y., Deng, Q., Zhao, X., Qiu, C., Xia, S., Liu, H., 2022. *Promotion effect of AM fungi and rhizobia co-inoculations on alfalfa (Medicago Sativa L.) growth.* Rhizosphere, 22: 100-537.

Xie, M.M., Zou, Y.N., Wu, Q.S., Zhang, Z.Z., Kuča, K., 2020. *Single or dual inoculation of arbuscular mycorrhizal fungi and rhizobia regulates plant growth and nitrogen acquisition in white clover.* Plant, Soil and Environment, 66(6): 287-294.