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## Efficiency of alfalfa seed priming and rhizobial-based liquid inoculants in an *Eutric Cambisol*

Aneta BUNTIĆ<sup>1</sup>, Olivera STAJKOVIĆ-SRBINOVIĆ<sup>1</sup>, Magdalena KNEŽEVIĆ<sup>1</sup>,  
Nataša RASULIĆ<sup>1</sup>, Vladan UGRENOVIĆ<sup>2</sup>, Djordje KUZMANOVIĆ<sup>1</sup>, Dušica DELIĆ<sup>1</sup>

<sup>1</sup>Institute of Soil Science, Department of Microbiology  
Teodora Drajzera 7, 11000 Belgrade, Serbia  
E-mail: anetabuntic@gmail.com

<sup>2</sup>Institute of Soil Science, Department of Soil Amelioration and Erosion  
Teodora Drajzera 7, 11000 Belgrade, Serbia

### Abstract

In the last few decades, peat-based bioinoculants have been proven as successful in alfalfa (*Medicago sativa* L.) seeds inoculation. However, the use of liquid inoculants or pre-inoculated seeds will greatly facilitate the sowing of alfalfa as a small seed legume. Therefore, this study evaluated the effect of four liquid formulations on the survival of *Sinorhizobium (Ensifer) meliloti* strain 218 during long-term storage. Sodium-alginate (SA) and polyvinylpyrrolidone (PVP) did not affect the growth of *S. meliloti* strain 218 and showed a significant positive effect on its survival in liquid bioinoculants up to 240 days of storage. The effect of seeds pre-inoculation on the efficiency of nitrogen (N) fixation in alfalfa plants in test tube and a two-year field experiment was measured. The field experiment was conducted in 2018 and 2019 in an *Eutric Cambisol*. The dry weight of alfalfa shoots and total N content significantly increased in pre-inoculated seeds with all media, compared to the control treatments without pre-inoculation and adding mineral N. In both years of cultivation, the highest total shoot dry weight was achieved by seeds pre-inoculation with SA and PVP 3 months before sowing. Furthermore, in these treatments, shoot dry weight significantly increased compared to the treatment with mineral N in the 2<sup>nd</sup> (2019) year. The results of the research showed that rhizobial bioinoculants with SA and PVP could be used as liquid bioinoculants as well as for successful pre-inoculation of alfalfa seeds.

Key words: rhizobia, *Medicago sativa*, liquid bioinoculants formulation, pre-inoculation, *Sinorhizobium meliloti*.

### Introduction

During the last decades, the excessive and improper use of chemical fertilisers and pesticides has contributed to the contamination and disturbance of natural balance in agricultural soils. The use of fertilisers based on different types of microorganisms (*Rhizobium* spp., *Azotobacter* spp., *Bacillus* spp., *Pseudomonas* spp., etc.) has emerged as an effective technology for enhancing the productivity and sustainability of agricultural systems (Andrews et al., 2011; Bargaz et al., 2018; Chinnaswamy et al., 2018).

Rhizobia are soil bacteria from the family of Leguminosae that can establish symbiosis with plant roots and fix atmospheric nitrogen (N) significant to the plant (Biswas, Gresshoff, 2014). Consequentially, the application of rhizobial bioinoculants (biofertiliser) can improve the N content and crop yield in an environmentally friendly manner (Delić, 2014). In addition, all this contributes to an increase in soil biodiversity (Ugrenović et al., 2020). This is of particular importance in organic production, where it is desirable to feed the plants through

soil ecosystems rather than using soluble fertilisers (Council Regulation (EC) No 834/2007). Rhizobial inoculants are also cheaper than inorganic fertilisers; therefore, their application brings fewer financial risks when used as a source to improve the productivity of leguminous crops (Naseer et al., 2019). Conventional microbial bioinoculants are commercially available in the form of a solid fertiliser commonly based on carriers such as peat, clay or coal (Malusá et al., 2012).

However, there are several disadvantages of solid microbiological fertilisers resulting in unpredictable field performance. Uneven distribution of microorganisms on solid carrier particles can lead to an uneven fertilisation effect on crops. The variability in quality and composition of peat and similar carriers should also be considered, as differences in its acidity could severely affect the viability of microorganisms (Malusá et al., 2012). Solid bioinoculants give the greatest effect when the seeds are inoculated just before sowing. However, in today's agricultural practice, farmers resort to application

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of already inoculated seeds in order to avoid seeds inoculation in the field. Therefore, the seed producers offer already inoculated seeds but at a higher price than uninoculated ones. This requires producers to inoculate the seeds a few months in advance during seed finishing processing.

On the other hand, the longevity of microorganisms on pre-inoculated seeds is questionable, as the number of viable bacterial cells decreases during the storage time. Due to the aforementioned disadvantages of solid biofertilisers, the use of liquid bioinoculants has emerged as a more efficient solution (Puwanto et al., 2019). Liquid bioinoculants ensure prolonged survival of microorganisms, which consequently indicates a longer shelf life of the product (Sehrawat et al., 2015). Seeds pre-inoculation also allows easier handling and transport, and growers do not need to worry about rushing and inoculating the seeds at the day of sowing (Sandini et al., 2019). One of the most critical steps are choosing the right carrier for a new bioinoculant formulation, its combination with the microorganisms in the basal medium as well as the selection of a highly effective strain (Bargaz et al., 2018). Bioinoculants for seeds pre-inoculation formulated on carriers such as sodium-alginate (SA), agar (A) and polyvinylpyrrolidone (PVP) are proven to have a great potential, mostly due to their ability to gradually release fertiliser in the soil during the time (Sampson et al., 2016; Bargaz et al., 2018; Ramli, 2019). Furthermore, these carriers do not interfere with the survival of rhizobial cells in the biofertilizer.

Alfalfa (*Medicago sativa* L.) is grown worldwide as a forage crop due to a high biomass yield, high nutritive value and digestibility. The importance of this crop is also related to the release of N into the soil and the improvement of the soil structure for future crops (Nešić et al., 2005; Kazemi Khaleedi et al., 2019). Alfalfa can establish a symbiosis with *Sinorhizobium meliloti* and fix atmospheric N significant to the plant. Alfalfa is a small-seeded leguminous plant, and artificial seeds inoculation with solid peat rhizobial bioinoculants can pose some difficulties such as seeds sticking and uneven sowing. Therefore, application of liquid bioinoculants or using pre-inoculated seeds would highly facilitate the sowing of alfalfa. In our previous study (Buntić et al., 2019), pre-inoculation of alfalfa seeds with liquid rhizobial bioinoculants (combination of agar and *S. meliloti* strain L3Si) one month before sowing enhanced the quality of alfalfa crops.

The aim of the experiment was to develop a liquid rhizobial bioinoculant for pre-inoculation of alfalfa seeds. Different media formulations with *S. meliloti* strain 218 were tested. The growth and survival of this strain under different storage temperature conditions were evaluated over a period of 240 days. The effect of pre-inoculation of alfalfa seeds by selected bioinoculants on the efficiency of N fixation in alfalfa plants was also evaluated in test tube and field experiment.

## Materials and methods

*Rhizobium* culture was prepared using efficient nitrogen (N) fixing *Sinorhizobium meliloti* strain 218 for alfalfa (*Medicago sativa* L.) cultivar K28 seeds. It was selected from the Department of Microbiology, the Institute of Soil Science collection (ISS WDCM375-Collection of Bacteria), Serbia. The *S. meliloti* strain 218 was grown in Erlenmeyer flasks in yeast mannitol broth (YMB): mannitol (10 g L<sup>-1</sup>), K<sub>2</sub>HPO<sub>4</sub> (0.5 g L<sup>-1</sup>), MgSO<sub>4</sub> × 7H<sub>2</sub>O (0.2 g L<sup>-1</sup>), NaCl (0.1 g L<sup>-1</sup>), CaCO<sub>3</sub> (0.2 g L<sup>-1</sup>) and fresh yeast extract (100 mL, 30 g L<sup>-1</sup>), on a rotary shaker (125 rpm) at 28°C temperature for 48 h (Buntić et al., 2019).

### Preparation of liquid rhizobial bioinoculants.

The basal medium for the formulation of liquid inoculants (LI) contained all components of YMB, except the mannitol (10 g L<sup>-1</sup>). Mannitol was added in concentration of 1 g L<sup>-1</sup> and presented one of the four tested formulations of inoculants for seeds pre-inoculation. Other three media were prepared by adding the following additives: liquid inoculant (LI) with agar (LI-A; 1 g L<sup>-1</sup>), LI with sodium-alginate (LI-SA; 1 g L<sup>-1</sup>) and LI with polyvinylpyrrolidone (LI-PVP; 10 g L<sup>-1</sup>). Liquid rhizobial bioinoculants were prepared by adding 1% inoculum of *S. meliloti* strain 218 (which was grown in YMB) to 50 mL of various media. All bioinoculants were placed in a rotary shaker (125 rpm) at 28°C temperature for 48 h.

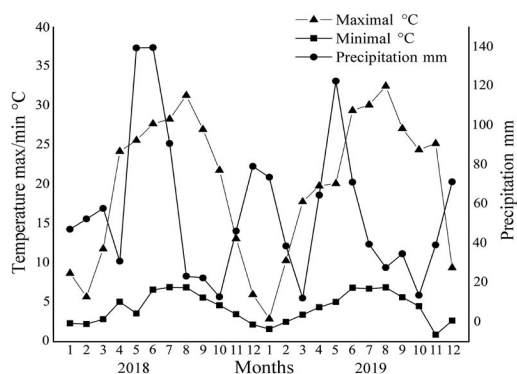
*Rhizobium* survival evaluation after prolonged storage. The survival of *S. meliloti* strain 218 in four different media formulations was tested. Prepared bioinoculants were stored at 4°C and 22°C temperatures for 240 days (8 months). The number of viable rhizobium cells after each 30 days of storage was determined by plating appropriate bioinoculant dilutions (10<sup>-6</sup>, 10<sup>-7</sup>, 10<sup>-8</sup> and 10<sup>-9</sup>) on YMA and incubation of 48 h. All measurements were performed in triplicate. Additionally, after the expiration of storage time, acidity (pH) was measured in all media.

*Pre-inoculation of alfalfa seeds and test tube experiment.* The pre-inoculation of alfalfa seeds was carried out by adding 250 µL of selected liquid inoculants (LI, LI-A, LI-SA and LI-PVP) to 2 g of seeds. Dried seeds were stored at 22°C temperature during a period of 0 (LI-0, LI-A0, LI-SA0 and LI-PVP0), 1 (LI-1, LI-A1, LI-SA1 and LI-PVP1), 2 (LI-2, LI-A2, LI-SA2 and LI-PVP2) and 3 (LI-3, LI-A3, LI-SA3 and LI-PVP3) months. Pre-inoculated seeds (10 seeds per treatment in triplicate) were sown every month in glass tubes with Jensen's medium agar. The pre-inoculation efficiency by examination of nodulation, plant height, shoot dry weight (SDW) and N content in SDW (N<sub>tot</sub>) was evaluated. N<sub>tot</sub> content was calculated by percentage of shoot N, which was determined from dried and ground plant samples (Horneck, Miller, 1988) using the Vario EL III Element Analyzer (Elemental Analysensysteme GmbH, Germany). The results were compared with two control treatments: non-inoculated seeds grown in Jensen's medium agar without (ØØ) and with (ØN) mineral N (N<sub>min</sub>) fertiliser in 0.05% KNO<sub>3</sub> (potassium nitrate) (Vincent, 1970).

*Field experiment.* For the field experiment, pre-inoculated alfalfa seeds with the media formulations of LI, LI-PVP and LI-SA and stored during zero (0), one (-1), two (-2) and three (-3) months were selected. The experiment was established in 2018 in the field, where alfalfa had not been sown so far, located in moderate-continental climate zone, 32 km from Belgrade in the village of Mala Ivanča (N 49°37'612", E 46°95'29") in *Eutric Cambisol* (WRB, 2014) and monitored for two (2018–2019) years.

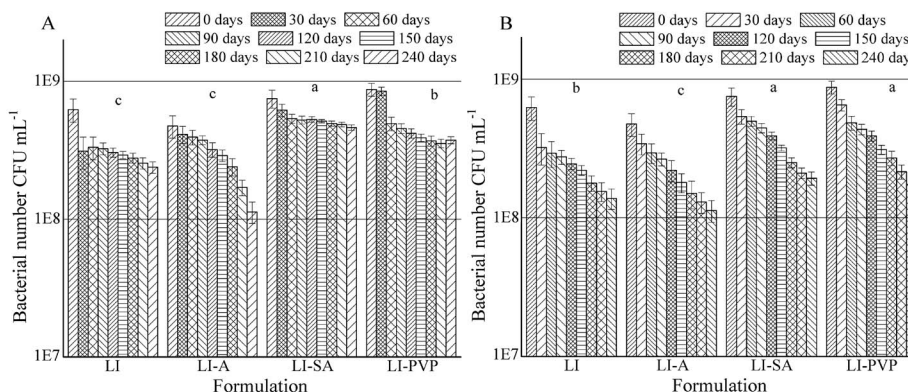
According to the data of the Meteorological Observatory Sopot, Republic Hydrometeorological Service of Serbia, average monthly air temperature (minimal and maximal) and precipitation for the experimental period are presented in Figure 1. Average monthly maximal and minimal temperatures for the two-year experimental period were 21.83°C and 7.91°C, respectively. Average sum of precipitation for this period was 657 mm.

Basic soil fertility parameters were: pH<sub>KCl</sub> 6.22, pH<sub>H2O</sub> 7.10, organic C 2.11%, available P 22.40 mg 100 g<sup>-1</sup>, available K 26.17 mg 100 g<sup>-1</sup>, N content 0.298% and C to N ratio 11.06. Two control treatments (with non-inoculated seeds) were also provided without (ØØ) and with (ØN) N<sub>min</sub> fertiliser application. The experiment was laid out in a completely randomized design in



**Figure 1.** Air temperature and precipitation for the region of the field experiment (according to the Republic Hydrometeorological Service of Serbia)

three replicates (for all treatments including controls). The alfalfa was planted in 1 m<sup>2</sup> plots at a sowing rate of 20 kg ha<sup>-1</sup>. The experimental field was not irrigated, fertilisers and pesticides were not used. During 2018 and 2019, three times a year, alfalfa biomass was collected (cut) at the full-bloom stage, and SDW and the N content in SDW (N<sub>tot</sub>) were determined. Each cutting of whole plots was carried out manually by cutting the plant at a height of 3–5 cm. Plant shoots were dried in an oven at 70°C temperature to constant weight, and the average dry weight per treatment was calculated. N<sub>tot</sub> content was calculated by percentage of shoot N (explained above).



*Note.* A – agar, SA – sodium-alginate, PVP – polyvinylpyrrolidone; treatments followed by the same letter are not significantly different ( $P > 0.05$ ); the bars above the columns denote the standard deviation (SD).

**Figure 2.** Survival of *Sinorhizobium meliloti* strain 218 in four different liquid inoculants (LI) formulations during storage at 4°C (A) and 22°C (B) temperatures

in LI (Figure 2A) after 240 days of storage at 4°C temperature. At higher storage temperature, the survival of *S. meliloti* strain 218 was lower and it ranged from 25.73% in LI-SA to 20% in LI-PVP.

ANOVA showed significant differences ( $P < 0.001$ ) in the number of viable bacterial cells between media formulation during storage at both temperatures. There were no differences in the changes in the number of bacteria between the LI and LI-A and LI-SA and LI-PVP treatments during storage at 4°C and 22°C temperatures, respectively. In addition, the pH values remained unchanged in all media formulations and were between 7.2 and 7.9 after the expiration of storage time. However, in all treatments, the number of viable bacterial cells of *S. meliloti* strain 218 was still above the number given by local regulation (Official Gazette of the Republic of Serbia Nos. 30/2017-13 and 31/2018-86).

**Pre-inoculation effect on alfalfa seeds.** In test tube experiment, the effect of pre-inoculation of alfalfa

**Statistical analysis.** To examine the differences between each media formulation, the effect of pre-inoculation of alfalfa seeds was evaluated by one- and two-way analysis of variance (ANOVA) followed by post hoc Duncan's test. All data represented are the mean  $\pm$  standard deviation (SD) of three replicates ( $n = 3$ ) of each treatment. The data were processed by statistical software package SPSS, version 17.0 (SPSS Inc., USA).

## Results

**Survival of *Sinorhizobium meliloti* strain 218 in liquid media under different storage conditions.** The effect of three additives: agar (A), sodium-alginate (SA) and polyvinylpyrrolidone (PVP), in liquid rhizobial bioinoculants on the viable cell count of *S. meliloti* strain 218 after 48 h of incubation was tested (Figure 2).

The viable cell counts ranged from  $4.75 \times 10^8$  (LI-A) to  $8.75 \times 10^8$  (LI-PVP) cell mL<sup>-1</sup> (presented as 0 days in Figure 2). In addition, the effects of different storage time and storage temperatures on the survival rate of *S. meliloti* strain 218 in four liquid formulations were monitored. Compared to the initial number of viable bacterial cells in all formulations, the highest decrease of viable cells of *S. meliloti* strain 218 was in LI-A treatment at both storage temperatures; it was slightly higher than  $1 \times 10^8$  cell mL<sup>-1</sup>. The number of viable rhizobia declined slightly in LI-SA and LI-PVP treatments during storage time from 60 to 240 days at 4°C temperature. Maximum cell survival ranged from 61.73% in LI-SA to 38.08%

seeds with four different liquid rhizobial bioinoculants on the plant nodulation, plant height, shoot dry weight (SDW) and N content in SDW (N<sub>tot</sub>) was estimated (Table 1).

The nodulation test with pre-inoculated seeds stored up to 3 months showed nodulation in all tested treatments on alfalfa roots. The percentage of nodulation for the seeds inoculated on the day of sowing and pre-inoculated seeds stored for 1, 2 and 3 months were 100%, except three (LI-3, LI-A3 and LI-A2) treatments. Using agar as supplement in liquid rhizobial bioinoculants for seeds coating and their storage more than 1 month was not satisfactory.

Applied treatments had a significant effect ( $P < 0.001$ ) on the plant height, SDW and N<sub>tot</sub> (Table 1). Compared to the control treatment without N ( $\emptyset$ ), significant increase of SDW using the pre-inoculated seeds stored for up to 3 months before sowing was observed in all pre-inoculated treatments. Generally, the SDW increased 2.57–4.53-fold compared to the control treatment ( $\emptyset$ ), except LI-3, LI-1, LI-A3, LI-A1, LI-

**Table 1.** The effect of seeds pre-inoculation on alfalfa growth and its N-fixing efficiency during test tube experiment

Treatment	Nodulation %	Plant height cm plant <sup>-1</sup>	SDW mg plant <sup>-1</sup>	N <sub>tot</sub> mg plant <sup>-1</sup>
LI-3	90	17.80 ± 1.06 bc	11.53 ± 0.28 i	0.42 ± 0.03 f
LI-2	100	17.73 ± 1.57 bcd	13.77 ± 0.25 f	0.54 ± 0.03 d
LI-1	100	16.87 ± 1.47 b-e	12.51 ± 0.02 g	0.49 ± 0.02 f
LI-0	100	17.33 ± 2.23 b-e	13.67 ± 0.23 f	0.55 ± 0.02 d
LI-A3	80	14.63 ± 0.85 de	9.95 ± 0.02 j	0.35 ± 0.02 h
LI-A2	90	15.50 ± 1.05 cde	13.72 ± 0.25 f	0.50 ± 0.02 e
LI-A1	100	14.33 ± 1.04 e	11.35 ± 0.29 i	0.49 ± 0.01 ef
LI-A0	100	15.92 ± 1.94 cde	12.43 ± 0.04 g	0.55 ± 0.02 d
LI-PVP3	100	18.67 ± 2.08 bc	14.58 ± 0.01 d	0.47 ± 0.02 ef
LI-PVP2	100	19.17 ± 3.40 b	14.24 ± 0.03 e	0.48 ± 0.01 ef
LI-PVP1	100	19.67 ± 1.53 b	11.43 ± 0.02 i	0.62 ± 0.01 c
LI-PVP0	100	26.10 ± 2.13 a	24.14 ± 0.29 a	1.08 ± 0.02 a
LI-SA3	100	17.50 ± 0.70 bcd	14.70 ± 0.27 d	0.47 ± 0.01 ef
LI-SA2	100	17.93 ± 0.83 bc	11.989 ± 0.04 h	0.33 ± 0.02 h
LI-SA1	100	17.83 ± 1.26 bc	12.82 ± 0.02 g	0.38 ± 0.02 g
LI-SA0	100	18.13 ± 0.91 bc	15.99 ± 0.03 c	0.61 ± 0.01 c
∅∅	0	6.83 ± 1.26 f	5.32 ± 0.02 k	0.09 ± 0.01 i
∅N	0	26.50 ± 1.80 a	21.43 ± 0.03 b	0.79 ± 0.02 b
One-way ANOVA		19.78***	1767.54***	428.97***

Note. Liquid inoculant (LI) without additives: LI-A – with agar, LI-PVP – with polyvinylpyrrolidone, LI-SA – with sodium-alginate; seeds storage: zero (0), one (1), two (2) and three (3) months; control ∅∅ – without N, ∅N – with N<sub>min</sub> (0.05% KNO<sub>3</sub>); SDW – shoot dry weight; N<sub>tot</sub> – N content in SDW; results were represented as mean ± SD; mean values followed by the same letter in the columns are not significantly different ( $P > 0.05$ ); one-way ANOVA shows  $F$  values; \*\*\* – significant at  $P < 0.001$ .

A0, LI-PVP1, LI-SA2 and LI-SA1 treatments. Most treatments gave N<sub>tot</sub> content between 0.468 (LI-SA3) and 0.616 (LI-PVP1) mg plant<sup>-1</sup> N. The highest N<sub>tot</sub> content of 1.08 mg plant<sup>-1</sup> N was obtained in LI-PVP0 treatment.

Two-way ANOVA showed that media formulation and storage time of seeds as well as their interactions significantly influenced plant height, SDW and N<sub>tot</sub> content (Table 2).

**Table 2.** The results of two-way ANOVA for plant height, shoot dry weigh (SDW) and total nitrogen (N<sub>tot</sub>) content of alfalfa plants

	df	Sum of square			Mean square			$F$ -value		
		Plant height	SDW	N <sub>tot</sub>	Plant height	SDW	N <sub>tot</sub>	Plant height	SDW	N <sub>tot</sub>
Media formulation	3	204.69	113.49	0.35	68.23	37.83	0.12	24.92***	1196.20***	375.81***
Storage time	3	39.91	153.08	0.52	13.30	51.03	0.17	4.86**	1613.50***	567.01***
Media formulation × storage time	9	76.94	190.70	0.43	8.55	21.19	0.05	3.12**	669.99***	156.00***
Error	32	87.62	1.01	0.01	2.74	0.03	0.0003			
Total	47	409.17	458.28	1.31						

Significant at \*\* –  $P < 0.01$  and \*\*\* –  $P < 0.001$ ; df – degree of freedom

all pre-inoculated treatments was significantly higher compared to the control treatment without N (∅∅) (Tables 3 and 4). In the 1<sup>st</sup> year of alfalfa cultivation in the field, SDW<sub>tot</sub> for inoculated treatments ranged between 6.82 (LI-0) and 7.42 (LI-SA3) t ha<sup>-1</sup>, respectively. The highest values of SDW<sub>tot</sub> were given in LI-SA3 in the 1<sup>st</sup> year (Table 3) and in LI-PVP in the 2<sup>nd</sup> year (Table 4). On the other hand, SDW<sub>tot</sub> in all treatments was lower compared to the control treatment with N<sub>min</sub> (∅N) (Table 3). According to two-way ANOVA, the medium formulation and storage time significantly influenced SDW at the 2<sup>nd</sup> and 3<sup>rd</sup> cutting, while their interaction showed no significance. Further, in the 2<sup>nd</sup> cutting, both factors and their interaction showed significant effect ( $P < 0.05$ ) on N<sub>tot</sub> content.

In the 2<sup>nd</sup> year, SDW<sub>tot</sub> for inoculated treatments ranged between 9.97 (LI-0 and LI-SA1) and 11.43 (LI-PVP3) t ha<sup>-1</sup>. The SDW<sub>tot</sub> of alfalfa was significant higher in LI-PVP3 (11.43 t ha<sup>-2</sup>) and LI-SA3 (11.38 t ha<sup>-2</sup>) compared to the control treatment (∅∅) as well as compared to the treatment with N<sub>min</sub> (∅N) (9.64 t ha<sup>-2</sup>) (Table 4). Pre-inoculation up to 2<sup>nd</sup> months before the sowing as well as inoculation on the day of sowing (1<sup>st</sup> and 2<sup>nd</sup> cutting) gave satisfactory results showing no significant differences in

comparison with the control treatment (∅N) using liquid rhizobial bioinoculants with SA and PVP. According to the tube experiment, where nodulation was decreased and shoot height and SDW value were lowest (Table 1) as well as the reduced survival of bacteria over prolonged time of storage in both temperatures (Figure 2), LI-A treatments were not included in the field experiment.

In the field experiment, in both experimental years, total SDW for all cuttings (SDW<sub>tot</sub>) per year of

comparison with the control treatment (∅N) using liquid rhizobial bioinoculants with SA and PVP.

In the 2<sup>nd</sup> year of alfalfa cultivation, the N<sub>tot</sub> content was higher 14% in LI-SA3 and 17% in LI-PVP3 compared to the control treatment with N<sub>min</sub> (∅N). In this regard, the pre-inoculation of alfalfa seeds with new rhizobial bioinoculants of these two formulations gave the highest yield and quality of alfalfa. The medium formulation was significant at the 1<sup>st</sup> and 3<sup>rd</sup> cutting for SDW, while their interaction with storage time showed significance only for the 1<sup>st</sup> cutting in the 2<sup>nd</sup> year of alfalfa cultivation. The storage time showed significance only in the 3<sup>rd</sup> cutting for SDW, while the individual factors and their interaction were not significant for N<sub>tot</sub> content in all cuttings. If the comparison is made between years, the total SDW was higher in the 2<sup>nd</sup> year than in the 1<sup>st</sup> one (Tables 3–5). In addition, all treatments showed higher values for the SDW<sub>tot</sub> and N<sub>tot</sub> content than control without N (∅∅) in the two-year average (Table 5).

ANOVA revealed significant effect of treatments, years and the treatment and year interaction on the SDW<sub>tot</sub> and N<sub>tot</sub> content (Table 5). According to two-year average for SDW<sub>tot</sub> and N<sub>tot</sub> content, the best treatment was LI-SA3 followed by LI-PVP3 treatment.

**Table 3.** The effect of seeds pre-inoculation on field alfalfa growth and its N-fixing efficiency in the 1<sup>st</sup> year (2018) of the experiment

Treatment	Cutting						
	1 <sup>st</sup>		2 <sup>nd</sup>		3 <sup>rd</sup>		
	SDW g m <sup>-2</sup>	N <sub>tot</sub> g m <sup>-2</sup>	SDW g m <sup>-2</sup>	N <sub>tot</sub> g m <sup>-2</sup>	SDW g m <sup>-2</sup>	N <sub>tot</sub> g m <sup>-2</sup>	SDW <sub>t ha<sup>-1</sup>tot</sub>
LI-3	138.33 ± 37.58 abc	5.87 ± 1.65 a-e	274.67 ± 5.03 cd	10.62 ± 1.46 b-e	196.67 ± 20.82 ef	8.23 ± 0.72 e	6.10 ± 0.17 cd
LI-2	152.67 ± 20.53 ab	7.59 ± 0.98 ab	269.33 ± 11.24 cde	11.22 ± 0.87 a-e	155.60 ± 41.33 g	8.16 ± 0.24 e	5.78 ± 0.56 de
LI-1	154.00 ± 15.10 ab	7.78 ± 1.01 a	250.20 ± 10.01 de	9.38 ± 0.52 def	236.67 ± 17.08 bcd	10.06 ± 0.91 bcd	6.41 ± 0.58 bc
LI-0	149.33 ± 5.03 ab	6.42 ± 1.02 a-d	206.67 ± 20.30 f	8.01 ± 0.51 f	189.44 ± 10.84 fg	8.13 ± 0.32 e	5.45 ± 0.08 de
LI-PVP3	142.00 ± 19.29 abc	6.49 ± 0.89 a-d	290.20 ± 10.01 bc	12.23 ± 0.84 abc	249.33 ± 22.03 abc	9.87 ± 1.12 d-e	6.82 ± 0.44 bc
LI-PVP2	160.00 ± 64.71 ab	6.45 ± 2.94 a-d	263.33 ± 15.28 cde	9.76 ± 2.59 c-f	236.00 ± 6.93 bcd	10.12 ± 1.24 bc	6.59 ± 0.24 bc
LI-PVP1	111.00 ± 16.46 bc	4.94 ± 0.89 c-e	257.60 ± 8.51 de	10.33 ± 1.04 c-f	202.13 ± 21.26 def	8.32 ± 1.44 de	5.71 ± 0.46 de
LI-PVP0	108.67 ± 5.77 bc	4.50 ± 0.57 de	269.60 ± 13.8 cde	11.49 ± 0.83 a-d	213.87 ± 14.89 c-f	8.93 ± 0.82 cde	5.92 ± 0.63 de
LI-SA3	170.67 ± 14.05 a	7.36 ± 1.54 abc	305.7 ± 19.25 ab	13.43 ± 2.08 a	265.33 ± 8.08 ab	10.87 ± 0.72 ab	7.42 ± 0.32 ab
LI-SA2	148.67 ± 11.12 ab	7.11 ± 0.45 abc	273.87 ± 21.17 cd	10.59 ± 0.96 b-e	228.53 ± 28.11 b-e	10.28 ± 0.90 bc	6.51 ± 0.85 bc
LI-SA1	141.00 ± 6.08 abc	6.06 ± 0.70 a-c	238.67 ± 18.63 e	8.90 ± 1.91 ef	239.07 ± 23.38 bcd	9.76 ± 0.86 b-e	6.19 ± 0.09 cd
LI-SA0	124.00 ± 48.04 abc	5.02 ± 2.17 b-e	276.73 ± 17.55 bcd	12.14 ± 1.08 abc	205.00 ± 18.03 def	8.29 ± 0.69 de	6.06 ± 0.23 cde
ØØ	96.33 ± 7.09 c	3.81 ± 0.37 e	130.33 ± 10.00 g	4.77 ± 0.75 g	117.93 ± 16.62 h	4.48 ± 0.35 f	3.45 ± 0.22 f
ØN	173.33 ± 15.28 a	7.77 ± 0.72 a	322.13 ± 33.03 a	12.86 ± 0.96 ab	284.67 ± 17.47 a	12.06 ± 0.88 a	7.80 ± 0.40 a
One-way ANOVA	2.270*	2.750*	25.869***	8.636***	12.979***	12.769***	15.567***
Two-way ANOVA							
Medium formulation	1.452 ns	2.628 ns	3.557*	3.579*	8.879**	1.493 ns	2.450 ns
Storage time	1.750 ns	2.423 ns	4.109*	3.215*	3.481*	3.048*	8.434**
Medium formulation × storage time	1.082 ns	1.078 ns	2.471 ns	2.861*	1.701 ns	2.552*	1.257 ns

Note. Explanations under Table 1; results were represented as mean ± SD; significant at \* –  $P < 0.05$ , \*\* –  $P < 0.01$  and \*\*\* –  $P < 0.001$ , ns – not significant; one- and two-way ANOVA shows  $F$  values.

**Table 4.** The effect of seeds pre-inoculation on field alfalfa growth and its N-fixing efficiency in the 2<sup>nd</sup> year (2019) of the experiment

Treatment	Cutting						
	1 <sup>st</sup>		2 <sup>nd</sup>		3 <sup>rd</sup>		
	SDW g m <sup>-2</sup>	N <sub>tot</sub> g m <sup>-2</sup>	SDW g m <sup>-2</sup>	N <sub>tot</sub> g m <sup>-2</sup>	SDW g m <sup>-2</sup>	N <sub>tot</sub> g m <sup>-2</sup>	SDW <sub>t ha<sup>-1</sup>tot</sub>
LI-3	203.47 ± 35.33 cd	7.98 ± 1.72 b	423.33 ± 23.09 bc	16.12 ± 1.42 a	373.33 ± 5.77 abc	15.30 ± 1.53 ab	10.00 ± 0.63 b-e
LI-2	213.33 ± 20.82 bcd	8.36 ± 0.79 ab	436.67 ± 15.28 abc	16.25 ± 3.14 a	383.33 ± 95.04 abc	16.99 ± 4.70 ab	10.33 ± 0.90 a-e
LI-1	230.00 ± 20.00 bc	9.40 ± 0.93 ab	426.67 ± 45.09 bc	14.92 ± 2.33 a	386.67 ± 15.28 abc	14.83 ± 0.97 ab	10.43 ± 0.76 a-d
LI-0	200.00 ± 20.00 cd	8.12 ± 0.82 b	430.00 ± 26.46 bc	16.98 ± 0.48 a	366.67 ± 11.55 abc	16.11 ± 1.78 ab	9.97 ± 0.32 c-e
LI-PVP3	264.13 ± 12.59 a	9.95 ± 0.41 a	466.67 ± 11.55 ab	18.84 ± 1.00 a	412.00 ± 26.46 a	17.72 ± 1.23 a	11.43 ± 0.12 a
LI-PVP2	246.67 ± 5.77 ab	9.94 ± 0.75 a	446.67 ± 45.09 abc	17.90 ± 2.34 a	406.67 ± 50.33 ab	16.09 ± 1.07 ab	11.00 ± 0.40 ab
LI-PVP1	231.33 ± 22.03 bc	9.61 ± 1.22 ab	443.33 ± 25.17 abc	16.40 ± 2.19 a	393.33 ± 5.77 abc	15.50 ± 0.46 ab	10.68 ± 0.52 a-d
LI-PVP0	223.33 ± 11.55 bc	9.10 ± 0.82 ab	400.00 ± 20.82 c	16.85 ± 1.61 a	389.33 ± 26.46 abc	15.51 ± 2.12 ab	10.13 ± 0.35 b-e
LI-SA3	249.67 ± 23.09 ab	9.11 ± 1.18 ab	473.33 ± 20.82 a	18.29 ± 1.54 a	415.33 ± 10.00 a	16.99 ± 3.02 ab	11.38 ± 0.67 a
LI-SA2	243.33 ± 15.28 ab	9.27 ± 0.06 ab	443.33 ± 5.77 abc	17.33 ± 3.92 a	370.00 ± 20.00 abc	13.32 ± 0.97 b	10.57 ± 0.45 a-d
LI-SA1	230.00 ± 20.00 bc	8.87 ± 0.86 ab	430.00 ± 20.00 bc	15.40 ± 2.02 a	336.67 ± 30.55 bcd	14.95 ± 1.85 ab	9.97 ± 0.53 c-e
LI-SA0	227.37 ± 4.56 bc	8.69 ± 0.73 ab	435.00 ± 15.28 bc	16.77 ± 1.76 a	410.00 ± 36.06 a	15.59 ± 1.40 ab	10.72 ± 0.51 a-c
ØØ	106.67 ± 0.58 e	5.60 ± 0.54 c	246.67 ± 5.77 d	9.10 ± 10.5 b	190.00 ± 20.00 e	7.82 ± 1.03 c	5.43 ± 0.26 f
ØN	225.07 ± 8.72 bc	8.74 ± 0.93 ab	425.33 ± 25.17 bc	16.07 ± 2.56 a	313.33 ± 23.09 d	12.31 ± 0.94 b	9.64 ± 0.53 e
One-way ANOVA	7.934***	4.254***	17.645***	3.481**	8.906***	5.402***	21.446***
Two-way ANOVA							
Medium formulation	8.214**	2.415*	0.619 ns	2.029*	3.926*	0.861 ns	2.596 ns
Storage time	2.066 ns	0.083 ns	1.364 ns	0.435 ns	1.539 ns	1.052 ns	2.575 ns
Medium formulation × storage time	1.365 ns	0.055 ns	3.907**	0.216 ns	0.752 ns	0.260 ns	1.770 ns

Note. Explanations under Table 1; results were represented as mean ± SD; significant at \* –  $P < 0.05$ , \*\* –  $P < 0.01$  and \*\*\* –  $P < 0.001$ , ns – not significant; one- and two-way ANOVA shows  $F$  values.

**Table 5.** The results of two-way ANOVA for average yield of total shoot dry weight (SDW<sub>tot</sub>) of alfalfa and total nitrogen (N<sub>tot</sub>) content during the experimental years (2018–2019)

Treatment	SDW <sub>tot</sub> t ha <sup>-1</sup>		N <sub>tot</sub> kg ha <sup>-1</sup>					
LI-3	8.05 ± 2.22 bcd		320.60 ± 85.45 bc					
LI-2	8.06 ± 2.64 bcd		342.85 ± 107.75 abc					
LI-1	8.42 ± 2.55 bc		331.85 ± 82.85 bc					
LI-0	7.71 ± 2.60 cd		318.85 ± 103.08 c					
LI-PVP3	9.13 ± 2.37 ab		375.50 ± 97.79 ab					
LI-PVP2	8.80 ± 2.55 ab		351.30 ± 103.34 abc					
LI-PVP1	8.20 ± 2.25 bcd		325.50 ± 88.73 bc					
LI-PVP0	8.03 ± 1.83 bcd		331.90 ± 77.57 bc					
LI-SA3	9.40 ± 2.27 a		380.25 ± 67.12 a					
LI-SA2	8.54 ± 2.46 bc		339.50 ± 83.09 abc					
LI-SA1	8.08 ± 2.27 bcd		319.70 ± 79.47 c					
LI-SA0	8.39 ± 2.35 bc		332.50 ± 70.82 bc					
∅∅	4.44 ± 1.11 e		177.90 ± 53.46 d					
∅N	8.72 ± 0.93 ab		349.05 ± 24.17 abc					
Year								
2018	6.17 ± 1.07 b		257.47 ± 47.90 b					
2019	10.12 ± 1.46 a		399.28 ± 58.97 a					
	df	Sum square	Mean square	F-value	df	Sum square	Mean square	F-value
Treatment	13	99.026	7.617	32.099***	13	158184.89	12168.07	16.017***
Year	1	316.863	316.863	1335.253***	1	405143.52	405143.52	533.284***
Treatment × year	13	18.883	1.453	6.121***	13	35903.32	2761.79	
Error	56	13.289	0.237		56	42544.01	759.71	3.635***
Total	83	448.062			83	641775.75		

Note. df – degree of freedom; results were represented as mean ± SD; significant at \*\*\* –  $P < 0.001$ .

## Discussion

Different microorganisms have shown unequal tolerance to various additives in liquid rhizobial bioinoculants. Choosing the appropriate material for maintaining a high number of microorganisms in the liquid medium is crucial for the development of a new microbial biofertiliser. In addition, these materials should not be toxic to microbes, be environmentally safe, should have a neutral or easily adjustable acidity (pH) and be available locally at a reasonable price (Bargaz et al., 2018; Buntić et al., 2019). Further, the physical and chemical properties of applied additives should protect bacterial cells against desiccation, sedimentation and cell death (Cortés-Patiño, Bonilla, 2015).

Liquid inoculant with sodium-alginate (LI-SA) or with polyvinylpyrrolidone (LI-PVP) had no detrimental effect on the growth of the rhizobium *Siborhizobium meliloti* strain 218 (Figure 2). They showed no negative rhizobial culturing conditions compared to the yeast mannitol broth (YMB) as liquid inoculant. On the other hand, agar showed a slightly negative effect on the growth of *S. meliloti* strain 218. Previous studies on the effect of different additives on the growth of microorganisms in the amended medium showed that agar and SA had negative effect on different rhizobia species such as *S. meliloti* L3Si, *Siborhizobium fredii* HH103, *Bradyrhizobium japonicum* USDA110, *Azorhizobium caulinodans* IRBG23 and *Mesorhizobium ciceri* USDA2429 (Hartley et al., 2013; Buntić et al., 2019). In contrast, in this experiment, SA was proven to be a good additive to the basal medium of *S. meliloti* strain 218 as well as PVP. In addition, Sehrawat et al. (2015) using PVP at the concentrations of 1% and 2% in liquid inoculants showed that this additive did not adversely affect the growth of *Rhizobium* strain MB703. In these media, the growth of rhizobium was also greater than in the YMB.

During the experiment, in all media formulations, the number of viable rhizobia (*S. meliloti* strain 218) decreased with increasing storage time up to 240 days. That was expected, as these additives cannot be used by rhizobial cells as an energy source. In fact, their main role in the liquid medium is to support cell growth and survival (Leo Daniel et al., 2013). Maintaining the

higher viable count during storage period is important and limiting factor for the commercialization of liquid inoculants (Trivedi et al., 2005; Buntić et al., 2019).

According to the local legislation, during storage time, the number bacterial cells in the liquid bioinoculant must not drop below  $1 \times 10^8$  cell mL<sup>-1</sup> (Buntić et al., 2019; Puwanto et al., 2019). Figure 2 represents the effect of the storage time on the viability of *S. meliloti* strain 218 in various formulations. The greatest preservation of viable cells occurred in media formulations with LI-SA and LI-PVP during 240 days of storage. This may be due to sticky consistency of these additives, which gives them adhesive properties. Further these properties may protect the cells from desiccation and drying. PVP is capable of detoxifying fermentation media by complexing with the phenolic-type and limiting the retention of toxins in the media (Girisha et al., 2006; Sehrawat et al., 2015). Additionally, this additive has protective property known as colloidal stabilization (Sehrawat et al. 2015).

During the storage period, temperature is also a limiting factor for the efficiency of the bioinoculants. Storage of bioinoculants at low temperatures is generally more suitable for bacterial survival, which was confirmed during this experiment (Figure 2). However, storage at lower than room temperature is not practical and requires additional cost (Deaker et al., 2004). In previous study of Cortés-Patiño and Bonilla (2015), inconsistent results regarding rhizobia survival were obtained. Survival is also influenced by the choice of bacterial strain and additives. SA is proven as good additive in the bioinoculant formulation providing successful survival during 60 days of storage at 28°C temperature (Cortés-Patiño, Bonilla, 2015). In contrast to *S. meliloti* strain 218, rhizobial *S. meliloti* strain L3Si showed lower survival (only 150 days at room temperature) in the medium formulation with the same concentration of SA (Buntić et al., 2019). Sehrawat et al. (2015) studied the survival of the *Rhizobium* strain MB703 in mannitol-supplemented liquid bioinoculants with PVP at room temperature. This medium formulation also gave greater survival of the strain used than the one with glycerol and gum arabic.

In the current study, presence of SA and PVP in media formulations provided suitable conditions for *S. meliloti* strain 218 survival, which was confirmed by unchanged pH values and protection of cells from



elevated temperature in relation to 4°C. Thus, the shelf life of *S. meliloti* strain 218 in a mixture of enrichment with these additives in relation to liquid inoculant (LI) was successfully extended.

Compared to the traditional inoculation, the technique of pre-inoculation of legume seeds brings several advantages: absence of seeds inoculation during sowing facilitates the work of farmers in the field, lowers the overall inoculants' production costs, reduces the amount of liquid inoculants used and diminishes the use of N<sup>min</sup> fertiliser (Delić et al., 2011; Buntić et al., 2019). Pre-inoculation of alfalfa seeds with SA and PVP as coating polymers can be justified, as the nodule number per plant did not decrease with storage time. These additives create a suitable microenvironment for the rhizobium, but this cannot be said for agar as coating polymer. This reduction of nodulation was associated with dying of rhizobia over time (Buntić et al., 2019). Based on one-way ANOVA, it was observed that all applied treatments had a highly significant effect ( $P < 0.001$ ) on all tested parameters: number of nodules, plant height, SDW and N<sup>tot</sup> content. The greatest influence had media formulations with SA and PVP: LI-PVP0, LI-SA0, LI-SA3, LI-PVP3 and LI-PVP2. However, the test tube experiment included small amount of pre-inoculated seeds, and these observations had to be confirmed in the field.

Field experiment showed similar results to those obtained in the tube experiment. The medium formulations with LI-SA and LI-PVP had an extremely significant effect on the tested parameters – SDW and N<sup>tot</sup> content. In particular, treatments LI-PVP3 and LI-SA3 gave satisfactory effect and confirmed the efficiency of designed novel rhizobial bioinoculants for pre-inoculation of seeds. Considering the two parameters together, the formulation with LI-SA in terms of alfalfa quality (N<sup>tot</sup> content) and the yield of SDW<sup>tot</sup> could be preferred (Table 5). In addition, due to the highly effective *S. meliloti* strain 218 used in this experiment as the active agent of the bioinoculant, the quality of plants, whose seeds were inoculated before sowing using LI-SA and LI-PVP formulations, was better than the control treatment with N<sup>min</sup> (ØN) in the 2<sup>nd</sup> year of field experiment. It can be explained by the fact that a highly effective rhizobial strain was used in the microbial formulations. In addition, the formulation additives (carriers) used did not interfere with the growth of the rhizobium and did not affect its effectiveness in processes of symbiotic N fixation.

Differences in the alfalfa SDW<sup>tot</sup> and N<sup>tot</sup> content between the experimental years were expected. This is also correlated with the fact that the genetic potential of alfalfa depends on its life stage and age, as alfalfa is a perennial crop (Tucak et al., 2017). The higher yield obtained in the 2<sup>nd</sup> year is in accordance with studies by other authors (Tucak et al., 2017; Tlahig et al., 2021). They have shown that maximum yield potential is achieved in the 2<sup>nd</sup> year of alfalfa cultivation.

Furthermore, 2018 and 2019 were the warmest years in Serbia since meteorological measurements were performed. The amount of precipitation was moderate for both years but was higher in 2018 (RHSS [http://www.hidmet.gov.rs/ciril/meteorologija/klimatologija\\_godisnjaci.php](http://www.hidmet.gov.rs/ciril/meteorologija/klimatologija_godisnjaci.php)). The total SDW in the 2<sup>nd</sup> year was higher than in the 1<sup>st</sup> one, although in 2019 it was warmer and there was less precipitation. The yield obtained in both experimental years is in line or somewhat lower compared with previously published result for normal temperature conditions (Katić et al., 2011). However, according to Tucak et al. (2017), alfalfa yield reaches 15.65–22.26 t ha<sup>-1</sup> even in dry years. Nevertheless, the use of the medium formulation of inoculants with SA and PVP for pre-inoculation of alfalfa seeds gave satisfactory results of alfalfa cultivation improving the yield compared to the control treatment and can be a good choice of alfalfa cultivation at high temperatures.

## Conclusion

The additives sodium-alginate (SA) and polyvinylpyrrolidone (PVP) did not affect the growth of *Sinorhizobium meliloti* strain 218 and showed a significant positive effect on its survival in liquid rhizobial bioinoculants during prolonged storage up to 240 days (8 months). In the field experiment, the alfalfa shoot dry weight (SDW<sub>tot</sub>) and N content in SDW (N<sub>tot</sub>) (average for two years) significantly increased in all pre-inoculated treatments compared to the control. The pre-inoculation technique yielded good results using inoculant formulation with LI-SA3 followed by LI-PVP3. Designed liquid rhizobial bioinoculants with SA and PVP provided double benefits. They can be used for both inoculation and pre-inoculation. The pre-inoculation method facilitates seeds sowing as well as increases alfalfa yield and its quality (higher N content in alfalfa plants).

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## Liucernos sėklų apdorojimo ir skystų *Rhizobium* inokuliantų veiksmingumas pasotintame rudžemyje

A. Buntić<sup>1</sup>, O. Stajković-Srbinić<sup>1</sup>, M. Knežević<sup>1</sup>, N. Rasulić<sup>1</sup>, V. Ugrenović<sup>2</sup>,  
D. Kuzmanović<sup>1</sup>, D. Delić<sup>1</sup>

<sup>1</sup>Dirvožemio mokslo instituto Mikrobiologijos skyrius, Serbija

<sup>2</sup>Dirvožemio mokslo instituto Dirvožemio gerinimo ir erozijos skyrius, Serbija

### Santrauka

Keletą pastarųjų dešimtmečių mėlynžiedės liucernos (*Medicago sativa* L.) sėkloms inokuliuoti buvo naudojami durpių pagrindu pagaminti bioinokuliantai. Tačiau naudojant skystus inokuliantus arba iš anksto inokuliuotas sėklas, labai palengvėtų liucernų kaip smulkiasėklių pupinių augalų sėja. Tyrimo metu buvo vertintas keturių skystų preparatų poveikis *Sinorhizobium* (*Ensifer*) *meliloti* 218 kamieno išgyvenamumui ilgai laikant. Natrio alginatas (SA) ir polivinilpirolidonas (PVP) neturėjo įtakos *S. meliloti* 218 kamieno augimui, tačiau turėjo reikšmingą teigiamą poveikį jo išgyvenimui skystus bioinokuliantus laikant 240 dienų. Vertinta sėklų išankstinės inokuliacijos įtaka azoto (N) fiksacijos efektyvumui liucernos augaluose mėgintuvėliuose ir dvejų metų lauko eksperimento metu. Lauko eksperimentas buvo vykdytas 2018 ir 2019 m. pasotintame rudžemyje (*Eutric Cambisol*). Liucernos ūglių sausoji masė ir suminis N kiekis reikšmingai padidėjo iš anksto inokuliuotose sėklose visose terpėse, lyginant su kontroliniu variantu be išankstinės inokuliacijos ir nepridėjus mineralinio N. Didžiausią suminę ūglių sausąją masę abiem auginimo metais užtikrino sėklų išankstinė inokuliacija SA ir PVP iki sėjos likus trims mėnesiams; ūglių masės padidėjimas buvo reikšmingas, palyginti su apdorojimu mineraliniu N antraisiais (2019) tyrimo metais. Tyrimo rezultatai parodė, kad *Rhizobium* bioinokuliantai su natrio alginatu ir polivinilpirolidonu gali būti naudojami kaip skysti bioinokuliantai liucernų sėklų išankstiniam inokuliuvimui.

Reikšminiai žodžiai: išankstinė inokuliacija, *Medicago sativa*, *Rhizobium*, *Sinorhizobium meliloti*, skystųjų bioinokuliantų sudėtis.