Botanica SERBICA

(2024) 48 (2): 195–205

DOI: https://doi.org/10.2298/BOTSERB2402195K
journal homepage: botanicaserbica.bio.bg.ac.rs

Original Scientific Paper

Bio-based solution for improving plant growth under unfavourable conditions: Bacterial inoculants for bird's foot trefoil and orchardgrass grown in acid soil

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ABSTRACT:
The agricultural industry is constantly searching for new solutions to increase the productivity and nutritional value of crops under various conditions. Microbial inoculants have emerged as an alternative to traditional chemical fertilisers which could enhance crop productivity in acid soils - a major problem in modern agriculture. The aim of this research was to evaluate the effects of *Bacillus megaterium* and *Mesorhizobium* sp. on the seed germination of bird's foot trefoil and orchardgrass under low pH (*in vitro*), as well as the nutrient composition of plants grown in acid soil. A positive impact of bacterial inoculation on seed germination was observed at pH 5 and 6 for both plant species. The content of macro- and microelements was within the range of optimal values for both plant species. This research provides valuable insights into the potential benefits of using bacterial inoculants to improve the seed germination and nutrient composition of plants grown in acid soils.

Keywords:
acid soil, *Bacillus megaterium*, *Mesorhizobium* sp., *Dactylis glomerata*, *Lotus corniculatus*, nutrient composition

UDC: 582.736.3+582.542.11:581.186

Received: 20 March 2024
Revision accepted: 03 July 2024

INTRODUCTION

Soil acidification, driven by factors such as chemical fertiliser usage, changes in soil exploitation and industrial emissions, leads to various adverse environmental impacts, including the increased leaching of elements and heavy metals into the ground and surface water, reduced soil biodiversity, and increased greenhouse gas emissions (Prescott *et al.* 2021). Acid soils present significant challenge to modern agriculture, especially in regions characterised by high rainfall and intensive farming practices, as their low pH reduces the availability of essential plant elements, such as calcium, magnesium, and phosphorus (Ferguson *et al.* 2013). Consequently, this leads to decreased crop yields and reduced crop quality, ultimately resulting in economic losses for farmers (Fageria & Nascente 2014; Zhu *et al.* 2020).

Despite the fact that approximately 3.95 billion hectares of soil are classified as acid, microbial inoculants formulated specifically for these conditions have not yet received adequate attention from the scientific and agronomic communities (Kaur *et al.* 2019). Therefore, it is necessary to develop sustainable and cost-effective solutions to mitigate soil acidity. Enhancing forage growth on acid soils can improve the nutrient composition of plants, boosting both their quality and yield. This approach not only addresses the challenge of soil acidity, but also presents a sustainable solution for marginal soils. Microbial inoculants formulated from beneficial soil bacteria are becoming increasingly popular as a

sustainable alternative to conventional chemical fertilisers, primarily due to the ability of selected bacteria to increase the availability of elements to plants, degrade toxic compounds, act as biocontrol agents, and alleviate the negative impact of abiotic stress (Alori & Babalola 2018; Fira *et al*. 2018; Marković *et al*. 2023; Raspor *et al*. 2023). In contrast to chemical fertilisers, which often consist of inorganic compounds with potential negative effects on soil and water quality, bacterial inoculants are eco-friendly alternatives which can be tailored for specific crop or soil types (Buntić *et al*. 2021; Kumar *et al*. 2022). In addition, inoculants can enhance plants' natural defences against diseases and pests, leading to healthier and more resilient crops (Mataranyika *et al*. 2022; Shahwar *et al*. 2023). By improving soil structure and increasing soil organic matter, inoculants can contribute to the long-term health and productivity of agricultural soils. Overall, using microbial inoculants based on beneficial bacteria can be an effective and eco-friendly approach to improve crop production under unfavourable soil conditions, including soils with low pH.

Bird's foot trefoil (*Lotus corniculatus* L.) is a common forage crop which can grow on alkaline or acid soils. This plant species is highly tolerant to elevated concentrations of aluminium and manganese, as well as salinity (Casler & Undersander 2019). Additionally, it naturally occurs in various heavy metal-enriched soils, indicating its high adaptability to such conditions (Sujkowska-Rybkowska *et al*. 2020). Orchardgrass (*Dactylis glomerata* L.) is a perennial grass which is a desirable alternative to tall fescue (*Festuca arundinacea* Schreb). Due to its favourable structure, it is highly suitable for livestock feed as hay, both as a monoculture or in combination with alfalfa (Ćupina *et al*. 2014; Aiken *et al*. 2020). Although this grass can grow on low-fertility soils, it reaches its maximum production potential on nitrogen-rich soils. Despite their tolerance to acidity, the yield and quality of both bird's foot trefoil and orchardgrass could be severely impaired by an acid environment, consequently leading to reduced production and quality of fodder.

This research is the continuation of experiments which showed that the inoculation of bird's foot trefoil and orchardgrass (grown both in monoculture and mixture) with rhizobial (*Mesorhizobium* sp. 631oz) and non-rhizobial PGP bacterial strains (*Bacillus megaterium* DZK1Bh) has a beneficial effect on the yield and nitrogen content in plants grown in acid soil (Knežević *et al*. 2021). As acid soils are characterised by a deficiency of beneficial elements such as phosphorus, magnesium, and calcium, and an abundance of aluminium, iron, and manganese, the aim of this research was to obtain a wider perspective of the influence of bacterial inoculation on the content of macro- and microelements in bird's foot trefoil and orchardgrass grown in acid soil. Elucidating the effects of inoculating both legume and non-legume plant species on plant yield and quality will allow the selection of effective bio-inoculants for cultivating economically significant plants in acid soils.

MATERIALS AND METHODS

Bacterial strains. For this research, the following bacterial strains isolated from the root nodules of bird's foot trefoil were used: *Mesorhizobium* sp. 631oz and *Bacillus megaterium* DZK1Bh. The strains were previously genetically characterised based on the 16S rRNA sequences and deposited in the NCBI base under accession numbers: OK067380.1 (strain 631oz) and MZ031917.1 (strain DZK-1Bh). Both applied strains were characterised as acid-tolerant and have the ability to produce indole-3-acetic acid (IAA) (Knežević *et al*. 2021, 2022a). For the seed germination test and pot experiment, the strains were grown in appropriate growth media (nutrient broth (Torlak) for *B. megaterium* and yeast mannitol broth (mannitol: 10 g - Biolife; K_2HPO_4: 0.5 g - Centrohem; $MgSO_4$: 0.2 g - Alkaloid; NaCl: 0.1 g - Zorka; $CaCO_3$: 0.2 g - Centrohem; yeast extract: 5 g -Torlak, dH_2O: 1000 mL) for *Mesorhizobium* sp.) at 28°C on an orbital shaker until the concentration of approximately 10^9 CFU mL^{-1} was reached.

Seed germination test. In order to explore the ability of the strains to improve the germination of bird's foot trefoil and orchardgrass seeds in an acidic environment, a seed germination test was performed in Petri dishes on Yensen agar ($CaHPO_4$: 1 g - Hemos; $MgSO_4 \times 7H_2O$: 0.2 g - Alkaloid; NaCl: 0.1 g - Zorka; $FeCl_3$: 0.1 g - Lachner; solution of microelements: 1 mL; agar: 8 g - Torlak; dH_2O: 1000 mL) with pH values of 5 and 6, while Jensen agar with a pH value of 7 was used as the control. The seeds (*Lotus corniculatus* L. - variety K30 and *Dactylis glomerata* L. - variety K24) were obtained from the Institute for Forage Crops Kruševac, Serbia. For the seed germination test the seeds were surface sterilised as described by Vincent (1970). The surface-sterilised seeds of bird's foot trefoil and orchardgrass were then submersed in bacterial cultures (10^9 CFU mL^{-1}) for 15 min with agitation, air dried, and placed on Petri dishes (10 seeds on each Petri dish, in triplicate). For the control without bacterial inoculation, sterile dH_2O was used instead of the bacterial culture. The Petri dishes were kept in the dark place at room temperature (22–24°C) for 14 days. The germinated and un-germinated seeds were counted, and the results were expressed as final seed germination percentage (FG%) (Ali *et al*. 2015) and relative seed germination index (RSGI%) (Buntić *et al*. 2019), based on the following equations (Eq. 1–2):

$$FG\ (\%) = \frac{SGs}{PStot} \times 100 \qquad (1)$$

$$RSGI\ (\%) = \frac{SGs}{SGc} \times 100 \qquad (2)$$

Table 1. The treatments applied in the pot experiment set up

Plant species	Treatment	Repetitions (pots)
Lotus corniculatus monoculture	Ø	3
	ØN	
	T1: *Bacillus megaterium* DZK1Bh	
	T2: *Mesorhizobium* sp. 631oz	
	T3: *Mesorhizobium* sp. 631oz + *Bacillus megaterium* DZK1Bh	
Dactylis glomerata monoculture	Ø	
	ØN	
	T1: *Bacillus megaterium* DZK1Bh	
	T2: *Mesorhizobium* sp. 631oz	
	T3: *Mesorhizobium* sp. 631oz + *Bacillus megaterium* DZK1Bh	
Lotus corniculatus + *Dactylis glomerata* mixture	Ø	
	ØN	
	T1: *Bacillus megaterium* DZK1Bh	
	T2: *Mesorhizobium* sp. 631oz	
	T3: *Mesorhizobium* sp. 631oz + *Bacillus megaterium* DZK1Bh	

where *SGs-* are germinated seeds in the sample, *PStot-* total planted seeds, and *SGc-* germinated seeds in the control.

Pot experiment set-up. The pot experiment was set up as described in Knežević *et al*. (2021), by using the soil with an acidic reaction of pH 5.37 (1 mol L^{-1} KCl). The bird's foot trefoil and orchardgrass were grown in the monoculture (30 seeds per pot) and in the mixture (20 seeds of bird's trefoil and 10 seeds of orchardgrass per pot). Inoculation was done with *Mesorhizobium* sp. 631oz and *B. megaterium* DZK1Bh separately, and as a co-culture. At the same time, controls with no inoculation (Ø) and with conventional fertilisation (ØN - N/P/K: 60/100/100 kg ha^{-1}) were also included. Each treatment, including the controls (Ø and ØN) was evaluated in three independent repetitions (pots), and the experiment was set up in a completely randomisd design (Table 1). The pots were kept under greenhouse conditions for seven weeks, after which the plant material was harvested.

Chemical analysis of the soil and plant material. The total content of the microelements in the soil sample was evaluated using the method for determining the trace elements (As, Cd, Cr, Co, Cu, Pb, Mn, Ni, and Zn) with inductively coupled plasma atomic emission spectrometry (ICP - AES) extracted with aqua regia. The method was based on the standard methods SRPS ISO 11466 (2004) and ISO 22036 (2008). The content of elements in the soil extract was determined using the ICP-OES instrument Thermo iCAP 6300 duo on predefined main and auxiliary wavelengths.

The plant material harvested from the pot experiment was dried at 70°C until completely dry and finely ground on an IKA 11 basic mill. The plant material was then weighed and treated with concentrated HNO_3 (Zorka). The reaction mixture was heated with gentle reflux after 16 h at room temperature (22–24°C). After the loss of brown steam, 30% H_2O_2 (Zorka) was added in two doses. Then, the solution was filtered, and appropriate dilutions were prepared for analysis using the ICP-OES ThermoiCAP 6300 duo instrument at predefined main and auxiliary wavelengths. The following certified reference materials and certified standard solutions were used for the calibration of the ICP-OES ThermoiCAP 6300 duo instrument: Multistandard ICP solution IV (AccuStandard) and Standard As, ICP standard, 1000 µg mL^{-1} As (AccuStandard) (Westerman *et al*. 1990; Watson 1998).

Statistical analysis. The effects of bacterial inoculation on the seed germination parameters (RSGI and FG percentage) were evaluated by two-way ANOVA. The influence of inoculation on the content of the macro- and microelements in the plant material was evaluated using analysis of variance (ANOVA). Duncan's multiple range test was used to test any differences between the means. The obtained data were processed using SPSS statistical software (version 17.0, SPSS Inc., USA) and expressed as the mean ± standard deviation (SD).

RESULTS

For the bird's foot trefoil, the relative seed germination index (RSGI) ranged between 100% (*B. megaterium* DZK1Bh at pH 5 and pH 7) and 153.85% (*Mesorhizobium* sp. 631oz at pH 5), and for the orchardgrass between 105.56% (*Mesorhizobium* sp. 631oz at pH 5) and 138.99% (*B. megaterium* DZK1Bh at pH 5). In general, the treatment of bird's foot trefoil with the *Mesorhizobium* sp. 631oz inoculum exhibited better performance in terms of the RSGI% compared to the treatment with the

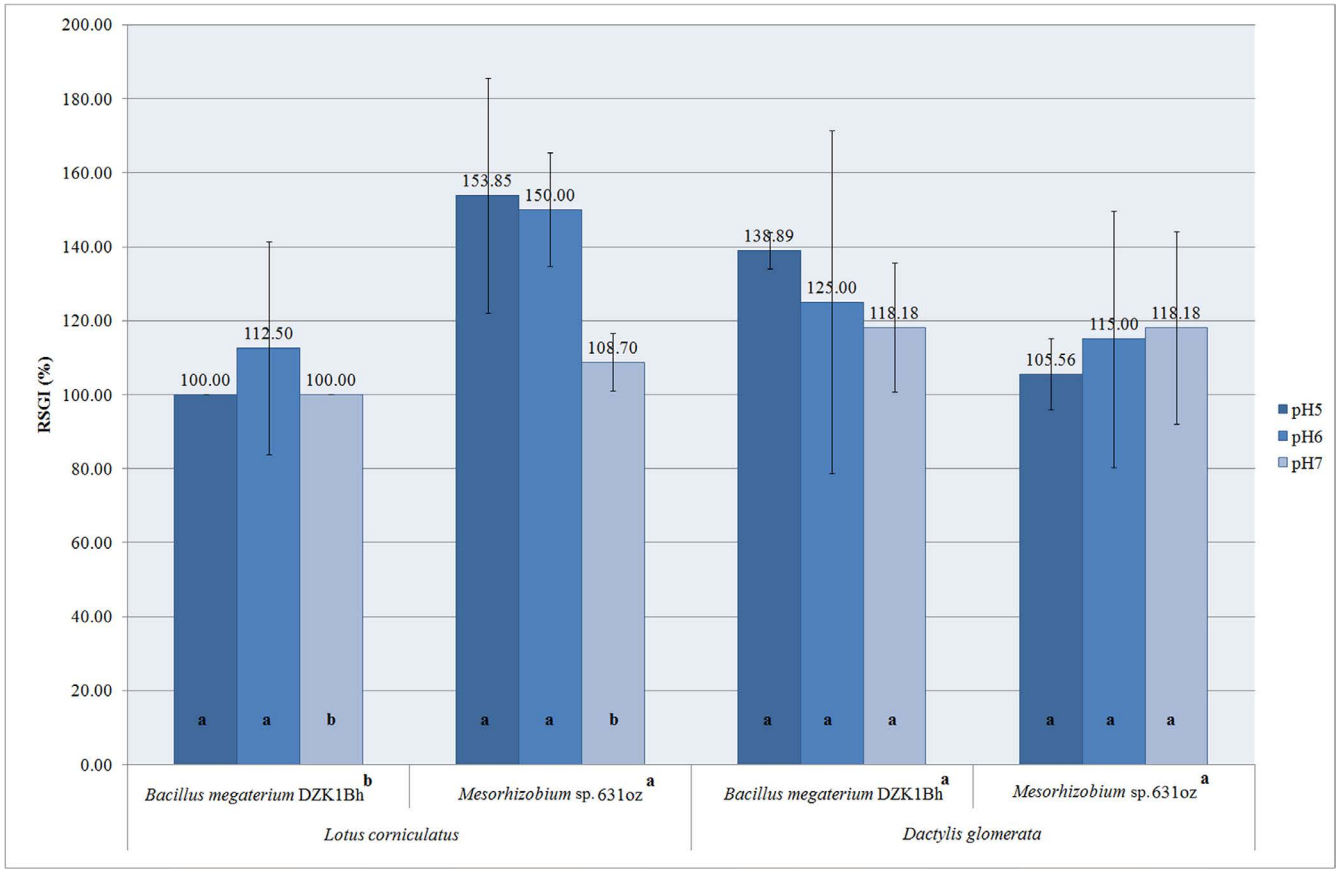

Fig. 1. The influence of bacterial inoculation on the relative seed germination index (RSGI) of *Lotus corniculatus* and *Dactylis glomerata*. Treatments marked with the same superscript letters are not statistically different according to two-way ANOVA. Bars indicating the RSGI% marked with the same superscript letters are not statistically different according to two-way ANOVA. The bars above the columns denote the standard deviation (SD).

B. megaterium DZK1Bh inoculum (Fig. 1). In the case of orchardgrass, no statistical differences were observed between the RSGI for both the applied inoculums (*B. megaterium* DZK1Bh and *Mesorhizobium* sp. 631oz). The effect of pH on the bird's foot trefoil RSGI was observed, with lower values of RSGI at pH 7 for both the applied treatments, compared to the values obtained at pH 5 and pH 6. Differences in pH did not affect the RSGI of the orchardgrass.

The final seed germination percentage (FG%) for the bird's foot trefoil ranged from 43.33% (control and *B. megaterium* DZK1Bh at pH 5) to 83.33% (*Mesorhizobium* sp. 631oz at pH 7), and from 60% (control at pH 5) to 86.67% (*Mesorhizobium* sp. 631oz and *B. megaterium* DZK1Bh at pH 7) for the orchardgrass (Fig. 2). For the bird's foot trefoil treatment with *Mesorhizobium* sp. 631oz significantly increased the FG%, while the effect of the *B. megaterium* DZK1Bh inoculum did not differ from the control. The treatment with *B. megaterium* DZK1Bh and *Mesorhizobium* sp. 631oz induced significantly higher FG% compared to the untreated control. The final germination percentage was the highest at pH 7, followed by pH 6 and pH 5 for the bird's foot trefoil. Similarly, the FG% for orchardgrass was higher at pH 7 and pH 6 than at pH 5. It is worth noting that for both the bird's foot trefoil and orchardgrass, the highest values of FG% were recorded at pH 7.

The results of the determination of the total concentrations of microelements in the soil used for the pot experiment are shown in Table 2. In the examined soil, the total concentrations of As, Cd, Co, Cr, Cu, Ni, Pb, and Zn were below the maximum allowed concentrations (MAC) according to the Official Gazette of the Republic of Serbia (Anonymous 1994, 2019), as well as Mn according to Schulin *et al.* (2010).

The concentrations of macroelements depended on the inoculum treatments, plant species, and cultivation methods (Table 3). For Ca and K, the values were the highest in T2: *Mesorhizobium* sp. 631oz, while the lowest values of K and Mg were recorded for T1: *B. megaterium* DZK1Bh (for the orchardgrass). The concentrations of Ca were higher in the bird's foot trefoil plants than the orchardgrass, regardless of the cultivation method. On the other hand, the concentrations of K, Mg, and P were relatively uniform. The concentrations of Ca were lower compared to the control and conventionally fer-

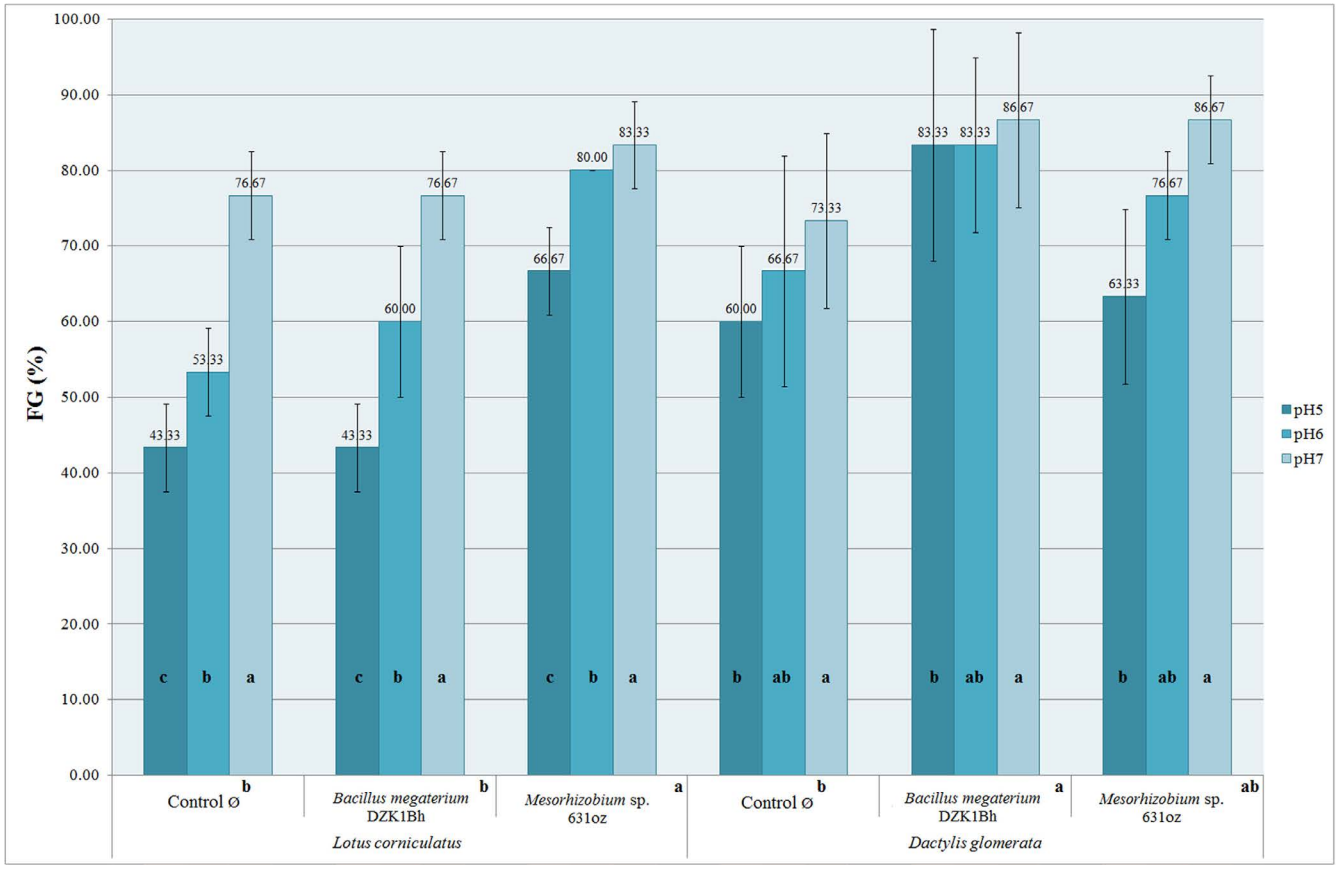

Fig. 2. The influence of bacterial inoculation on the final seed germination (FG%) of *Lotus corniculatus* and *Dactylis glomerata*. Treatments marked with the same superscript letters are not statistically different according to two-way ANOVA. Bars indicating different FG% marked with the same superscript letters are not statistically different according to two-way ANOVA. The bars above the columns denote the standard deviation (SD).

Table 2. The concentration of the total microelements in the soil used for the pot experiment

	Elements (mg kg^{-1})								
	As	Cd	Co	Cr	Cu	Mn	Ni	Pb	Zn
Total element concentration	4.55	0.55	9.61	31.40	8.01	506	20.50	20.00	58.40
MAC*	25	3	-	100	100	-	50	100	300

*MAC: maximum allowed concentration of elements in the soil, according to Anonymous (1994).

tilised control in the bird's foot trefoil plants cultivated in monoculture. The concentrations of K in the bird's foot trefoil plants were higher compared to the control sample in both cultivation methods. In the orchardgrass plants, the concentrations of K were lower in relation to the control sample (the orchardgrass in monoculture), while the highest value was recorded for T1: *B. megaterium* in the case of the orchardgrass in a mixture. The concentrations of Mg and P varied depending on the applied treatment.

The concentration of microelements in the plant material depended on the plant species and cultivation method (monoculture or mixture). In the case of the bird's trefoil plants, it was observed that the same treatments mainly had a similar influence on the concentration of microelements, regardless of the cultivation method (Tables 4 & 5). Concentrations of Co, Cr, Cu, Fe, Ni, and Zn were at their highest in the non-inoculated plants (Ø), while the concentration of Mn was the highest (62.31 mg kg^{-1}) under the influence of the T1: *B. megaterium* inoculum treatment (the bird's foot trefoil in monoculture). Concentrations of Pb (non-essential element) were at their highest under the effect of the T2: *Mesorhizobium* sp. treatment and the co-inoculation of *B. megaterium* + *Mesorhizobium* sp. (T3), and the control in the case of the bird's foot trefoil as a monoculture, while the highest value in the mixture was recorded for the co-inoculation of *B. megaterium* +

Table 3. The concentration of macroelements (Ca, K, Mg, and P) in the plant material

Plant species	Treatment	Macroelements (g kg^{-1})			
		Ca	K	Mg	P
Lotus corniculatus	Ø	15.62±0.20^{d}	32.37±0.21^{c}	4.56±0.02^{c}	3.47±0.07^{a}
	ØN	14.72±0.29^{e}	36.56±0.06^{b}	4.70±0.04^{c}	3.24±0.02^{ab}
	T1	16.33±0.02^{c}	40.53±0.13^{a}	5.04±0.03^{b}	3.47±0.09^{a}
	T2	19.08±0.20^{b}	27.49±0.07^{d}	5.30±0.10^{a}	2.95±0.04^{b}
	T3	19.59±0.08^{a}	22.70±0.10^{e}	4.93±0.05^{b}	3.31±0.01^{ab}
Lotus corniculatus (from the mixture)	Ø	15.61±0.28^{a}	35.41±0.10^{a}	4.97±0.19^{b}	2.98±0.11^{b}
	ØN	16.26±0.14^{a}	28.33±0.34^{d}	5.34±0.02^{a}	3.11±0.06^{a}
	T1	16.50±0.10^{a}	31.91±0.12^{c}	4.93±0.05^{b}	3.12±0.06^{a}
	T2	16.90±0.13^{a}	32.07±0.07^{c}	4.90±0.18^{b}	2.81±0.01^{c}
	T3	16.63±0.08^{a}	35.51±0.09^{b}	4.92±0.08^{b}	3.06±0.03^{bc}
Dactylis glomerata	Ø	4.64±0.02^{c}	42.29±0.04^{c}	5.01±0.01^{c}	2.56±0.01^{a}
	ØN	5.03±0.08^{b}	38.40±0.10^{d}	4.79±0.19^{d}	2.55±0.06^{a}
	T1	4.98±0.17^{b}	38.78±0.06^{d}	5.41±0.07^{b}	2.12±0.04^{b}
	T2	6.82±0.04^{a}	53.92±0.02^{a}	5.93±0.06^{a}	2.22±0.01^{ab}
	T3	6.70±0.10^{a}	44.83±0.24^{b}	5.97±0.02^{a}	2.44±0.04^{a}
Dactylis glomerata (from the mixture)	Ø	4.79±0.04^{c}	49.79±0.04^{a}	5.34±0.02^{a}	1.77±0.01^{c}
	ØN	4.74±0.02^{c}	39.68±0.18^{d}	4.77±0.05^{b}	2.15±0.05^{bc}
	T1	5.17±0.06^{a}	31.31±0.01^{e}	4.69±0.06^{c}	2.91±0.04^{a}
	T2	4.99±0.10^{b}	46.33±0.12^{b}	5.29±0.09^{a}	2.42±0.01^{b}
	T3	4.70±0.02^{c}	42.95±0.01^{c}	4.87±0.02^{b}	2.11±0.02^{bc}

T1: seeds treated with *Bacillus megaterium* DZK1Bh; T2: seeds treated with *Mesorhizobium* sp. 631oz; T3: seeds treated with *Bacillus megaterium* DZK1Bh + *Mesorhizobium* sp. 631oz; Ø: untreated control; NØ: conventional fertilisation without bacterial inoculation; a-e: the values in the column marked with the same letters are not statistically different according to Duncan multiple range test ($P \leq 0.05$);

Table 4. The concentration of microelements (Co, Cr, Cu, and Fe) in the plant material

Plant species	Treatment	Microelements (mg kg^{-1})			
		Co	Cr	Cu	Fe
Lotus corniculatus	Ø	0.18±0,04^{a}	1.96±0.04^{a}	10.57±0.06^{a}	460.12±9.25^{a}
	ØN	0.15±0.06^{ab}	1.67±0.12^{ab}	10.02±0.01^{ab}	254.01±6.87^{c}
	T1	0.10±0.01^{b}	1.18±0.01^{c}	8.60±0.01^{bc}	151.06±7.19^{d}
	T2	0.16±0.02^{ab}	1.40±0.02^{b}	9.86±0.00^{ab}	349.41±6.33^{b}
	T3	0.14±0.01^{ab}	1.64±0.06^{ab}	9.20±0.05^{b}	269.86±1.04^{c}
Lotus corniculatus (from the mixture)	Ø	0.25±0.03^{a}	1.86±0.04^{a}	10.53±0.06^{a}	363.28±3.98^{a}
	ØN	0.13±0.01^{b}	1.41±0.03^{b}	8.63±0.06^{a}	255.34±3.13^{b}
	T1	0.10±0.00^{b}	1.52±0.05^{b}	8.49±0.09^{c}	146.01±2.31^{c}
	T2	0.15±0.04^{b}	1.41±0.03^{b}	10.29±0.11^{a}	344.21±11.00^{a}
	T3	0.13±0.02^{b}	1.44±0.04^{b}	9.79±0.08^{b}	257.94±12.01^{b}
Dactylis glomerata	Ø	0.09±0.01^{a}	2.69±0.09^{a}	7.03±0.02^{ab}	174.74±8.11^{a}
	ØN	0.05±0.00^{a}	1.68±0.07^{b}	6.13±0.07^{b}	96.26±4.17^{bc}
	T1	0.05±0.02^{a}	1.70±0.04^{b}	5.50±0.13^{c}	75.10±4.12^{c}
	T2	0.06±0.01^{a}	1.81±0.05^{b}	5.39±0.06^{c}	90.28±5.42^{bc}
	T3	0.06±0.00^{a}	2.49±0.10^{a}	7.83±0.02^{a}	133.28±9.55^{b}
Dactylis glomerata (from the mixture)	Ø	0.39±0.03^{a}	1.08±0.02^{b}	5.02±0.03^{c}	82.09±6.64^{bc}
	ØN	0.06±0.01^{b}	2.13±0.02^{a}	6.88±0.04^{b}	157.94±8.20^{a}
	T1	0.05±0.02^{b}	1.21±0.01^{b}	5.85±0.01^{bc}	82.09±5.64^{bc}
	T2	0.07±0.02^{b}	2.59±0.04^{a}	7.93±0.03^{a}	157.94±8.20^{a}
	T3	BDL	1.49±0.02^{b}	6.33±0.05^{b}	92.96±5.863^{b}

T1: seeds treated with *Bacillus megaterium* DZK1Bh; T2: seeds treated with *Mesorhizobium* sp. 631oz; T3: seeds treated with *Bacillus megaterium* DZK1Bh + *Mesorhizobium* sp. 631oz; Ø: untreated control; NØ: conventional fertilisation without bacterial inoculation; a-d: the values in the column marked with the same letters are not statistically different according to Duncan multiple range test ($P \leq 0.05$); BDL: values below the detection limit of the method.

Table 5. The concentration of microelements (Mn, Ni, Pb, and Zn) in the plant material

Plant species	Treatment	Microelements (mg kg^{-1})			
		Mn	Ni	Pb	Zn
Lotus corniculatus	Ø	48.84±0.57^{b}	4.92±0.01^{a}	1.19±0.02^{ab}	70.55±2.14^{a}
	ØN	50.61±0.07^{b}	5.17±0.05^{a}	0.95±0.02^{b}	63.79±3.20^{b}
	T1	62.31±2.56^{a}	3.86±0.03^{c}	1.08±0.00^{b}	48.26±2.77^{c}
	T2	50.88±0.14^{b}	4.84±0.01^{ab}	1.42±0.01^{a}	59.96±4.15^{b}
	T3	44.77±0.74^{bc}	4.53±0.02^{b}	1.49±0.10^{a}	53.18±3.96^{bc}
Lotus corniculatus (from the mixture)	Ø	62.10±0.55^{a}	5.33±0.04^{a}	1.42±0.05^{a}	75.79±4.12^{a}
	ØN	48.23±0.83^{b}	4.99±0.05^{b}	0.95±0.03^{bc}	60.76±3.67^{b}
	T1	63.84±1.95^{a}	3.62±0.04^{c}	0.99±0.03^{bc}	47.50±2.98^{c}
	T2	49.62±0.10^{b}	4.71±0.02^{b}	1.15±0.02^{b}	60.83±3.14^{b}
	T3	42.86±1.35^{b}	4.25±0.01^{bc}	1.64±0.01^{a}	55.84±2.11^{c}
Dactylis glomerata	Ø	74.09±1.02^{bc}	2.63±0.08^{ab}	0.68±0.03^{a}	60.25±3.25^{a}
	ØN	79.94±0.99^{a}	2.04±0.01^{b}	0.55±0.01^{a}	58.79±2.75^{a}
	T1	73.84±1.01^{bc}	2.03±0.07^{b}	0.30±0.01^{b}	48.43±2.17^{bc}
	T2	77.45±0.90^{a}	1.89±0.03^{bc}	0.22±0.01^{b}	55.80±1.02^{a}
	T3	54.83±0.75^{c}	2.90±0.12^{a}	0.39±0.02^{b}	51.89±1.94^{b}
Dactylis glomerata (from the mixture)	Ø	57.15±0.74^{b}	1.59±0.05^{bc}	3.48±0.09^{a}	86.26±4.03^{a}
	ØN	75.37±0.93^{a}	2.44±0.02^{a}	0.23±0.04^{b}	60.59±3.28^{b}
	T1	75.99±1.30^{a}	1.82±0.01^{b}	0.36±0.05^{b}	54.41±2.10^{b}
	T2	65.43±0.87^{b}	2.88±0.02^{a}	0.34±0.07^{b}	56.09±4.12^{b}
	T3	61.61±1.12^{b}	2.04±0.07^{b}	0.22±0.02^{b}	50.05±3.97^{b}

T1: seeds treated with *Bacillus megaterium* DZK1Bh; T2: seeds treated with *Mesorhizobium* sp. 631oz; T3: seeds treated with *Bacillus megaterium* DZK1Bh + *Mesorhizobium* sp. 631oz; Ø: untreated control; NØ: NPK fertilisation without bacterial inoculation; a-c: the values in the column marked with the same letters are not statistically different according to Duncan multiple range test ($P \leq 0.05$).

Mesorhizobium sp. (T3) and the control. In addition to the concentrations of Mn, Ni, and Pb, a decreasing trend in the concentrations of microelements in the bird's foot trefoil plants (cultivated both in monoculture and in mixture) was observed under the influence of bacterial inoculation.

The T1: *B. megaterium* treatment induced the highest Mn concentration in the bird's foot trefoil plants in monoculture (62.31 mg kg^{-1}) and in the mixture (63.84 mg kg^{-1}). For the bird's foot trefoil in monoculture, the highest values of Ni concentration were found in the T2: *Mesorhizobium* sp. treatment, the control (Ø), and the control conventional fertilisation (ØN), while in the mixture, the highest concentration of Ni was recorded for the non-inoculated control (5.33 mg kg^{-1}).

In the orchardgrass plants, the concentrations of Co did not significantly differ. Concentrations of Pb and Zn were the highest in the non-inoculated control in both the cultivation methods. For monoculture, the concentration of Cu was at its highest when treated with T3: *B. megaterium* + *Mesorhizobium* sp. and in the control. For the mixture, the highest concentration of Cu was recorded for the T2: *Mesorhizobium* sp. treatment (7.93 mg kg^{-1}). The concentration of Fe in the orchardgrass monoculture was the highest for the non-inoculated control (174 mg kg^{-1}) and the T2: *Mesorhizobium* sp. treatment (157.94 mg kg^{-1}) in the mixture. The T2: *Mesorhizobium* sp. treatment induced the highest concentrations of Mn in the orchardgrass plants grown in the mixture, which were not significantly different from the control. The concentrations of Mn were at their highest in the nitrogen-treated control and in T2: *Mesorhizobium* sp. in the case of the orchardgrass grown in monoculture. No consistent trend for a specific treatment's influence on the concentration of Ni in the plant material could be observed. In the orchardgrass, the highest concentrations of Ni were recorded for the treatment with the co-inoculation of *B. megaterium* + *Mesorhizobium* sp. (T3) and the nitrogen-treated control (the orchardgrass in monoculture) and T2: *Mesorhizobium* sp. and the nitrogen-treated control (the orchardgrass from the mixture).

DISCUSSION

In conventional agriculture, the use of chemical fertilisers has facilitated successful crop production. However, the overuse of chemical fertilisers has become a significant problem in recent years. Their excessive application has led to a range of negative environmental impacts, including soil degradation, water pollution, and greenhouse gas emissions (Adesemoye *et al.* 2009; Tian *et al.* 2022). Furthermore, the long-term use of chemical fertilisers has been shown to negatively affect soil health by reducing its fertility and biodiversity (Sud 2020). It has been shown that inoculating legumes and non-legumes with microbial inoculants based on bacterial species belonging to

Bacillus and *Mesorhizobium* can have several beneficial effects in acid soil (Kaur *et al*. 2019). These inoculants can improve soil fertility and increase the availability of elements for plants, leading to increased crop yield (Jiménez-Gómez *et al*. 2018; Abulfaraj & Jalal 2021; Rakić *et al*. 2021). Additionally, *B. megaterium* can improve plant growth through the synthesis of substances which are essential for plants, while *Mesorhizobium* sp. can establish a symbiotic relationship with legumes, leading to improved plant growth and nitrogen fixation (Di Benedetto *et al*. 2017; Knežević *et al*. 2022b).

The present study investigated the effects of bacterial inoculation with *B. megaterium* and *Mesorhizobium* sp. on the germination of bird's foot trefoil and orchardgrass under low pH conditions *in vitro*. Additionally, it examined the impact of bacterial inoculation on the content of macro- and microelements in the plant material grown in pots with acid soil. The findings contribute to understanding the potential benefits of bacterial inoculation in promoting these plant species' growth and nutrient status in challenging soil conditions. The results demonstrated the positive influence of bacterial inoculation on the seed germination parameters for both bird's foot trefoil and orchardgrass. The strains *B. megaterium* and *Mesorhizobium* sp. exhibited favourable effects on the germination of seeds under low pH conditions, indicating their ability to enhance the early growth stages of these plants.

Although the values of final seed germination (FG%), both for the bacterial treatments and un-inoculated seeds, were the most significant at pH 7, the values of the relative seed germination index (RSGI%) were higher at lower pH values in the case of bacterial inoculation in comparison to the un-inoculated control. In general, *B. megaterium* DZK1Bh caused the highest increment of the RSGI% at pH 5 in comparison to the un-inoculated orchardgrass seeds. In contrast, the treatment of the bird's foot trefoil seeds with *Mesorhizobium* sp. 631oz showed the best result at pH 5. These results may be elucidated through the potential establishment of a symbiotic association between the applied rhizobial strain (*Mesorhizobium* sp. 631oz) and bird's foot trefoil. On the other hand, the non-rhizobial strain (*B. megaterium* DZK1Bh) demonstrated a more favourable effect on non-leguminous plant species, which can potentially be attributed to the synthesis of PGP substances, as the bacterial strains used in this research were previously determined as IAA producers (Knežević *et al*. 2021, 2022a). Overall, the ability of both applied strains to improve seed germination could be due to the production of bacterial IAA, as also suggested by other authors (Egamberdieva 2009; Fiodor *et al*. 2023). Similarly, it was demonstrated that acid-tolerant rhizobial strains (*Bradyrhizobium* sp. and *Sinorhizobium meliloti*) were more efficient in nitrogen fixation in symbiosis with legume cowpea, when the soil conditions were acidic, while IAA-producing *Bacillus firmus* produced prominent results in acidic environments on the non-legume rice (Kaur *et al*. 2019).

The ability of *B. megaterium* and *Mesorhizobium* sp. to promote the growth and development of bird's foot trefoil and orchardgrass under low pH conditions is noteworthy. These findings support the potential application of microbial inoculants as an alternative approach to enhance crop productivity under unfavourable conditions, including acid soils (O'Callaghan 2016; Sarkar *et al*. 2021). The positive effects observed on seed germination highlight the importance of early plant establishment, which is critical for overall plant performance (De Luis *et al*. 2008). Additionally, the specific mechanisms underlying the observed effects of bacterial inoculation should be elucidated to provide a comprehensive understanding of the interactions between the plant species, microbial strains, and environmental conditions (O'Callaghan 2022).

Further, this study examined the impact of bacterial inoculation on the content of macro- and microelements in the plant material (in pots). Soil acidification often leads to the accumulation of toxic elements, such as aluminium and manganese, which can harm plant growth and ultimately affect human health through the food chain (Singh *et al*. 2017). In addition, soil acidification can cause imbalances in soil microbial communities, which can in turn negatively impact soil health and crop productivity (Ferguson *et al*. 2013). The major constraint to plant growth in soils with low pH is the toxic effects of Al, Mn, and Fe, while beneficial elements such as Mo, P, Mg, and Ca become less available to plants (Kaur *et al*. 2019; Msimbira & Smith 2020). The excess of these elements in soils induces stronger stress than their deficiency (Kabata-Pendias 2004). The bioavailability of micro- and macroelements to plants is one of the most significant problems in agriculture. Currently, soil remediation is most commonly used to obtain neutral soil pH by liming and phosphate application (Kabata-Pendias 2004). However, these techniques do not always produce the expected outcomes. Although the soil used for the pot experiment had a low pH (5.37), the total content of microelements was acceptable, according to the Official Gazette of the Republic of Serbia (Anonymous 1994). The results revealed variable effects of bacterial inoculation on the nutrient composition of the plants grown in this soil. This suggests that the influence of bacterial inoculation on nutrient uptake and assimilation is complex and may be influenced by multiple factors, including the specific strains used and the soil characteristics (İpek *et al*. 2021). However, the content of both the macro- and microelements was within the range of optimal values (Kabata-Pendias 2010). In our previous research, the inoculation of bird's foot trefoil with *B. megaterium* DZK1Bh and *Mesorhizobium* sp. 631oz separately and a mixture of the two significantly

increased shoot dry weight compared with the untreated control, while in the case of orchardgrass, the mixture of *B. megaterium* DZK1Bh + *Mesorhizobium* sp. 631oz had the best effect on the plant shoot dry weight. In addition, the same research showed that all the treatments increased the total nitrogen content compared to the control (Knežević *et al*. 2021). Furthermore, the inoculation of bird's foot trefoil with *Mesorhizobium* sp. 631oz significantly increased the shoot dry weight and total nitrogen content of the plants grown in acid soil, and the uptake of macro- (P, K, Ca, and Mg) and microelements (Cu, Fe, Mn, Ni, Zn, and B) also increased (Knežević *et al*. 2022a). Other authors have also demonstrated that the inoculation of legume plants with rhizobial strains can cause differences in the concentration of elements such as N, P, K, Ca, and Mg in plants, depending on the plant variety or plant tissues (Francini *et al*. 2010; Bertins *et al*. 2021). Vučković *et al*. (2006) showed that the Ca content in non-inoculated bird's foot trefoil plants was higher than the values recorded in this research. In addition, concentrations of K and Mg were higher than those recorded in Vučković *et al*. (2006), while the concentration of P was similar. Accordingly, the concentrations of Ca, P, and Mg in plants of bird's foot trefoil grown in Greece obtained in a study by Koutsoukis *et al*. (2019) were lower than the findings in the current study. This could indicate that inoculation of bird's foot trefoil both by rhizobial and non-rhizobial strains could improve the nutrient uptake. Zetochova *et al*. (2020) showed that the different levels of effectiveness of bacterial inoculation could also be due to the differences in environmental factors such as the temperature and moisture of the soil as well as soil nitrogen content.

CONCLUSION

In conclusion, this study demonstrated the potential of bacterial inoculation with non-rhizobial (*Bacillus megaterium*) and rhizobial (*Mesorhizobium* sp.) strains in improving the germination of bird's foot trefoil and orchardgrass under low pH conditions. The effects of bacterial inoculation on the nutrient content of the plants in acid soil were variable, suggesting the need for further investigations to unravel the underlying mechanisms. These findings contribute to the knowledge of microbial-assisted strategies for sustainable agriculture and provide valuable insights for future research in enhancing plant growth and productivity in challenging soil environments.

Acknowledgements – This research was supported by the Ministry of Science, Technological Development and Innovation of the Republic of Serbia, contract No. 451-03-66/2024-03/200011 and 451-03-65/2024-03/200178.

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REZIME

Botanica SERBICA

Biološka rešenja za poboljšanje rasta biljaka u nepovoljnim uslovima: Inokulacija bakterijama žutog zvezdana i ježevice uzgajanih u kiselom zemljištu

Magdalena Knežević, Tanja Berić, Aneta Buntić, Zoran Dinić, Dušica Delić, Slaviša Stanković i Olivera Stajković-Srbinović

Poljoprivredna proizvodnja je u kontinuiranoj potrazi za novim rešenjima koja bi doprinela povećanju produktivnosti i poboljšanju nutritivne vrednosti useva u različitim uslovima. Mikrobni inokulanti su se pojavili kao alternativa tradicionalnim hemijskim đubrivima, koja predstavljaju veliki problem u savremenoj poljoprivredi, a što bi moglo da poveća produktivnost useva u kiselim zemljištima. Cilj ovog istraživanja bio je da se proceni efekat inokulacije sa *Bacillus megaterium* i *Mesorhizobium* sp. na klijavost semena žutog zvezdana i ježevice pri niskom pH (*in vitro*), kao i uticaj na nutritivni sastav ovih biljnih vrsta gajenih u kiselim zemljištima. Pozitivan uticaj bakterijske inokulacije na klijanje semena primećen je pri pH vrednostima od 5 i 6 kod obe biljne vrste. Sadržaj makro- i mikroelemenata bio je u optimalnom opsegu vrednosti kod obe biljne vrste. Ovo istraživanje pruža uvid u potencijalne koristi primene bakterijskih inokulanata za poboljšanje klijavosti semena i nutritivnog sastava biljaka gajenih u kiselim zemljištima.

Ključne reči: kiselo zemljište, *Bacillus megaterium*, *Mesorhizobium* sp., žuti zvezdan, ježevica, nutritivni sastav

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