Original paper DOI: 10.5937/ZemBilj2402014B

Improvement of yield and nutrient composition of orchardgrass in soils with increased nickel concentration by inoculation with *Bacillus* strains

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Abstract

The pollution of soil by potentially toxic trace elements (PTE) is increasing every year and has become a serious problem worldwide. Plant growth is influenced by numerous interactions with the environmental factors and organisms from the surrounding soil, including high concentration of PTE. The aim of this research was to evaluate the possibility of growing orchardgrass in soils with an increased Ni concentration and improvement of the plant quality in these stressful conditions using *Bacillus* inoculums. In addition, the plant growth promoting traits and the germination of inoculated *Dactylis glomerata* seeds with *Bacillus* inoculants was examined. The main goal was to examine the effects of inoculums on plant yield, nutrient composition, and trace elements concentration in orchardgrass biomass. The ability of bacterial isolates to tolerate high concentration of Ni as well as the potential of these bacteria to enhance the germination of orchardgrass seeds was tested in vitro on Petri dishes. A pot experiment was conducted in soil with elevated Ni concentration, and plant dry mass, as well as the content of micro and macro elements in plant material was determined. The results of the pot experiment showed the positive effect of bacterial inoculation on shoot dry weight of orchardgrass, while the nitrogen content in orchardgrass shoots was slightly increased under the influence of bacterial inoculation. Furthermore, the content of microelements in plant material was reduced in inoculated plants.

Keywords: Bacillus inoculants, *Dactylis glomerata*, soil remediation, trace elements, plant quality

Introduction

An essential task in the 21st century is rational and sustainable land use, with the goal of safe food production and biodiversity conservation (Raza et al., 2019). There are estimations that the food demand might increase from 30-35% to 56-62% between 2010 and 2050 to support population growth and eradicate global hunger (van Dijk et al., 2021). Food production will also depend on global climate change that will likely result in degradation and loss of arable agricultural land and more stressful conditions for crop cultivation caused by variations in annual rainfall, temperature fluctuations, heat waves, insect pest attacks, salinity, change in CO_2 and ozone levels, fluctuations of the sea levels (Raza et al., 2019). As this could put global food security at risk, finding effective yet sustainable ways for managing plant stress and improving crop yield, is of great importance for providing food for the growing world population in the changing climate (Kumar et al., 2019). Therefore, finding green and cost-effective alternative is crucial and use of microbial inoculants as bio-fertilizers instead of chemical fertilizers represents a promising and sustainable solution to this problem.

Plant growth under field conditions is influenced by numerous interactions with the environmental factors and organisms from the surrounding soil. Instead of talking about plants individually, a more appropriate term would be a holobiont which takes into account plant and its associated microbiome, also called phytomicrobiome (Backer et al., 2018). Among various rhizobacteria, *Bacillus* spp. are very prominent plant growth-promoting rhizobacteria (PGPR) possessing multiple PGP traits, including nitrogen fixation, mechanisms to increase nutrient availability, phytohormones production, siderophores, antimicrobial compounds, and hydrolytic enzymes, induced systemic resistance (ISR), and tolerance to abiotic stress (Setiawati et al., 2022). Another trait that also makes them good candidates for agricultural and biotechnological application is the ability to produce highly resistant endospores enabling survival under unfavorable environmental conditions, which could also be used to prolong the fertilizer shelf-life. Also, because of their ubiquitous nature and their natural presence in microflora of many plant species, most strains are considered non-phytopathogenic and safe for crops. In fact, numerous studies indicate beneficial effects of various *Bacillus* species on the growth and health of different plant species under normal as well as stressful conditions, including important vegetable crops and grasses (Khan et al., 2020; Masmoudi et al., 2021; Kalam et al., 2020; Lozo et al., 2022; Shin et al., 2021; Kumar et al., 2021; Tahir et al., 2019; Gagné-Bourque et al., 2015; Niu et al., 2016; Rakić et al., 2021) when applied as single inoculant or in the form of bacterial consortium. Also, bio-fertilizers based on different *Bacillus* strains have been registered and commercialized so far.

The pollution of soil by potentially toxic trace elements (PTE) is increasing every year and has become a serious problem worldwide. Sources of heavy metals can be divided into natural and anthropogenic (Alengebawy et al., 2021). Bacteria can affect metal bioavailability in soil and therefore increase or decrease uptake of heavy metals by plants. By secreting various organic acids and biosurfactants, PGPR mobilize metals and make them more available for plant uptake (Yang et al., 2018; Li et al., 2010). Mobilization of metals through biomethylation, forms methylated metal compounds with different solubility and toxicity and leads to metal volatization. PGPR have mechanisms to stabilize metals leading to decreased metal bioavailability, through processes of metal immobilization, precipitation, alkalization and complexation. PGPR can mediate oxidation-reduction processes of metals which result in chemical transformation of metals and change of their mobility which leads to reduced toxicity (Mushtaq et al., 2022). Through processes of biosorption and bioaccumulation, where metal pollutants adhere to cell surface or accumulate inside the bacterial cell, PGPR mediate detoxification of heavy metals and lower the detrimental effects of metals on plants (Wang et al., 2022).

Orchardgrass (*Dactylis glomerata*), also known as cocksfoot, is a perennial species from the *Poaceae* family native to Europe, North Africa, and parts of Asia, but has been introduced worldwide.

It stays productive for 4-8 years and can be harvested 4 times a year. Optimum growth conditions for orchardgrass include annual day temperature from 4.3 to 23.8ºC, and rich soils such as clays and loams with pH ranging from 4.5 to 8.2. It tolerates high temperatures, drought and shade, but it does not endure excessive humidity very well. Due to its good nutritive value and high yields, but also being less competitive and non-toxic compared to tall fescue, it is suitable for planting in mixture with other grasses and legumes, usually with alfalfa and red clover for hay or with white clover for grazing (Aiken et al., 2020, Heuze and Tran, 2015). It has a deep fibrous root and can also be used for soil erosion control, rehabilitation of overgrazed lands or lands disturbed by mining, and stabilization of soil that has been burned (Wang et al., 2023).

There is a small number of studies on how PGPR, and *Bacillus spp.* in particular, affect the growth and nutrient uptake of orchardgrass. Previous work of researchers from this group indicated a potential of different *Bacillus* species to promote growth of orchardgrass, applied as single inoculant or co-inoculated with other species (*Azotobacter* or *Mesorhizobium*) when grown in a single grass culture or as a mixture with legume plants (Stajković-Srbinović et al., 2016, Knežević et al., 2021). There is also limited data on the phytoremediation potential of orchardgrass or how rhizobacterial inoculation influence uptake of nutrients as well as potentially toxic elements. There are studies indicating that orchardgrass is suitable for phytostabilization as it was successfully grown on metal-contaminated, nutrient-poor mine soil with the highest concentration of metals accumulated in the roots (Visconti et al., 2020; Pogrzeba et al., 2019).

The aim of this research was to evaluate the possibility of growing orchardgrass in soils with an increased Ni concentration and improvement of the plant quality in these stressful conditions using *Bacillus* inoculums. In addition, the plant growth promoting traits and the germination of inoculated *D. glomerata* seeds with *Bacillus* inoculants was examined. The main goal was to examine the effects of inoculums on plant yield, nutrient composition, and trace elements concentration in orchardgrass biomass.

Materials and Methods

Sample materials

In this research, two bacterial inoculants were applied: *Bacillus halotolerans* Vig3NK2 and *Bacillus megaterium* DZK1Bh, previously identified by Knežević et al. (2021). Strain Vig3NK2 was isolated from rhizosphere soils of *Medicago sativa* L., while strain DZK1BH was isolated from root nodules of wild *Lotus corniculatus* L. plants. The soil used for the experiment was collected in 2022 from Makiš locality near Belgrade, Serbia.

Tolerance to nickel

Tolerance to nickel for strains was assessed on Nutrient agar (NA) plates (Somasegaran and Hoben, 1994). The NA plates had a range of nickel (II) sulfate heptahydrate of 50 -150 mg L^{-1} (Ni, pH 6). All media were autoclaved at 121°C for 20 min. Bacterial strains were inoculated on prepared media, incubated (28°C, 24 h) and all tests were done in three replications.

Seed germination tests

The ability of two *Bacillus* strains to improve germination of orchardgrass seeds in the presence of different concentrations of nickel was performed *in vitro* on Petri dishes. The experiment was done in a transparent sealed box in a light chamber (22°C, programmed at 16/8 h). An aliquot of 2 mL of overnight culture was added to the microtube with 40 seeds. The mixture was agitated (180 rpm, 5 min) and orchardgrass seeds (inoculated and uninoculated) were placed onto Jensen agar with and without the addition of different concentrations of Ni (0, 50, 100, and 150 mg L^{-1}). The experiment was conducted for 10 days in two independent repetitions and the results were averaged. The results were expressed by the relative seed germination (%) index (RSGI) (Buntić et al., 2017) calculated based on the following equation:

$$\mathrm{RSGI}(\%) = \frac{\mathrm{SG_s}}{\mathrm{SG_c}} \times 100 \qquad (1)$$

where SG_S is germinated seeds in samples, SG_C is germinated seeds in control.

Pot experiment

In each pot 730 g of gravel was used as the bottom layer and 1570 g of soil was added as a top layer. Approximately 0.1 g of *D. glomerata* seeds were inoculated with bacterial inoculums (>10^9 cells mL^{-1}) and placed evenly on the soil in each pot. Treatments used in pot experiment were as follows: T1: (DZK1BH inoculum), T2: (Vig3NK2 inoculum), ØØ: negative control (no fertilizer and no bacterial inoculum added), ØN: treated with recommended dosages of chemical fertilizers (N: 60 kg ha^{-1}, P: 100 kg ha^{-1} and K: 100 kg ha^{-1} and no bacterial inoculum added). Seeds were then covered with a thin layer of soil and pots were kept under greenhouse conditions during one season starting from May 2022. The experiment was set up with three replicates for each treatment and pots were placed in a completely randomized pattern. Plants were harvested before the plant blooming period with two harvests in total, the first in July 2022 and the second in October 2022. After the second harvest, root samples were also collected.

Soil properties

Soil analysis was performed in accordance with SRPS ISO 11464:2004. The basic soil chemical parameters were determined using the following methods: soil pH (SRPS EN ISO 10390:2022); available phosphorus and potassium by the AL method (Egnér et al., 1960); carbonate content (SRPS ISO 10693:2005); and organic matter content according to Kotzmann (Kotzmann, 1930). Concentrations of Ni and other microelements were determined using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (SRPS EN ISO 22036:2024) after the sample was digested with aqua regia according to ISO 54321:2020.

Plant material analysis

Plant and root material was dried at 65°C and grounded in a mill. CNS analyzer (Vario model EL III) was used to measure N (%) in the plant samples (Horneck and Miller, 1998; Miller, 1998). Macro- and micronutrient concentration was determined using THERMO iCAP 6300 Duo ICP-OES (Watson, 1998).

Results and Discussion

Basic soil parameters of soil used for pot experiments are shown in Table 1.

Table 1. Basic chemical properties of soil used for pot experiment

Basic chemical parameters	
pH (KCl)	7.35
pH (H_2O)	8.01
Soil organic matter (SOM) (%)	5.41
P_2O_5 (mg 100 g^{-1})	34.07
K_2O (mg 100 g^{-1})	27.06
$CaCO_3$ (%)	4.03

The soil was alkaline (pH (KCl) > 7.2) with elevated Ni concentrations, which were almost twice higher than the maximum allowed concentrations (MAC = 50 mg kg^{-1}) for this element (Official Gazette of RS, 1994). The content of other trace elements in the soil was lower than MAC. According to the obtained Ni concentration in the soil, the tolerance of applied *Bacillus* strains to the presence of three Ni concentrations in the medium was tested. Both strains, DZK1Bh and Vig3NK2 were able to grow at the concentration of Ni 50 and 100 mg L^{-1}, while bacteria did not grow at the highest Ni concentration of 150 mg L^{-1}.

RSGI gradually decreased with an increase of nickel concentration, in comparison to the control without nickel, which had higher values (Figure 1). Application of both bacterial inoculants increased the percentage of RSGI in all nickel treatments as well as in the control sample. In treatment with nickel concentration of 150 mg g^{-1}, RSGI was two times higher compared to the control sample with the same nickel concentration. Ndeddy Aka and Babalola (2015) showed a similar effect of brassica seeds germination using metal tolerant bacterial strains. Three applied bacterial strains including *Bacillus* (*Pseudomonas aeruginosa* KP717554, *Alcaligenes feacalis* KP717561, and *Bacillus subtilis* KP717559) increased germination index of brassica seeds in the presence of nickel, chromium and cadmium (Ndeddy Aka and Babalola, 2015).

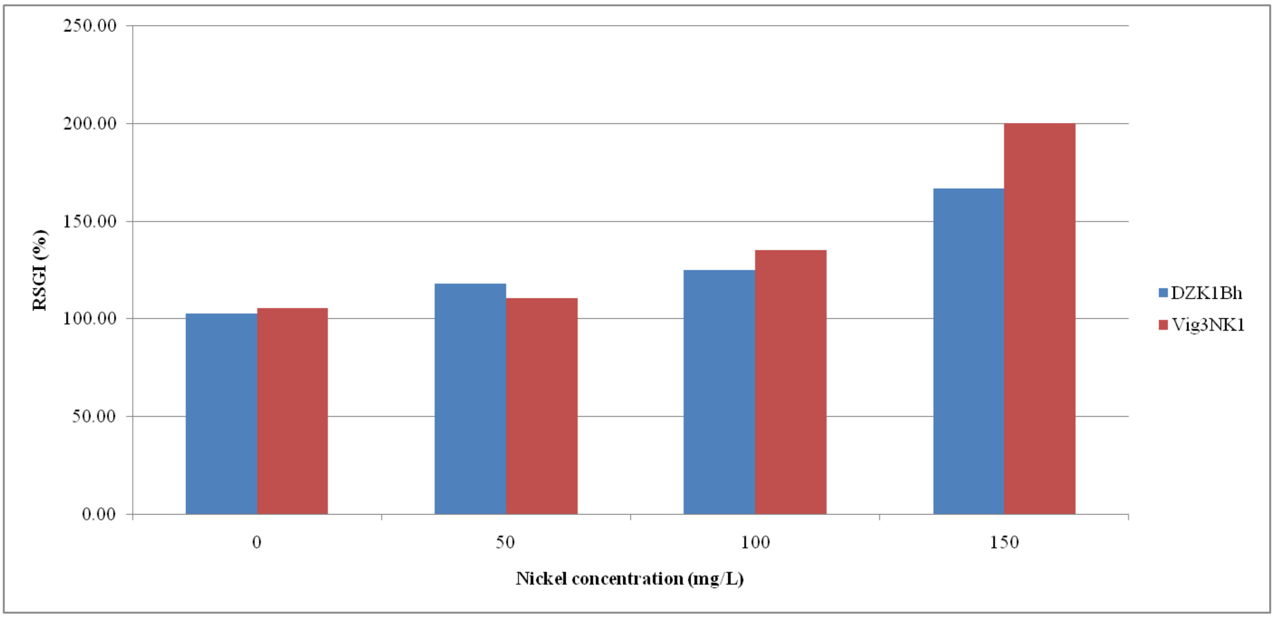

Figure 1. Relative seed germination index of inoculated orchardgrass seeds on different nickel concentrations. Data is presented as average values from three replicates.

The results of the pot experiment showed the positive effect of bacterial inoculation on the shoot dry weight of orchardgrass in both cuttings (Figure 2). Also, the increase in shoot dry weight was recorded for plant roots under the influence of bacterial inoculation. In the first cutting, inoculation by DZK1Bh induced an increase of shoot dry weight 1.61 times in comparison to the uninoculated control. Similarly, inoculation of orchardgrass by the same strain increased shoot dry weight 1.18 times in comparison to the uninoculated control. In addition, the same strain showed the best effect on root dry weight and increased it 1.58 times in comparison to the uninoculated control. Overall, inoculation of orchardgrass by strain DZK1Bh showed the best effect on root and shoot dry weight in both cuttings.

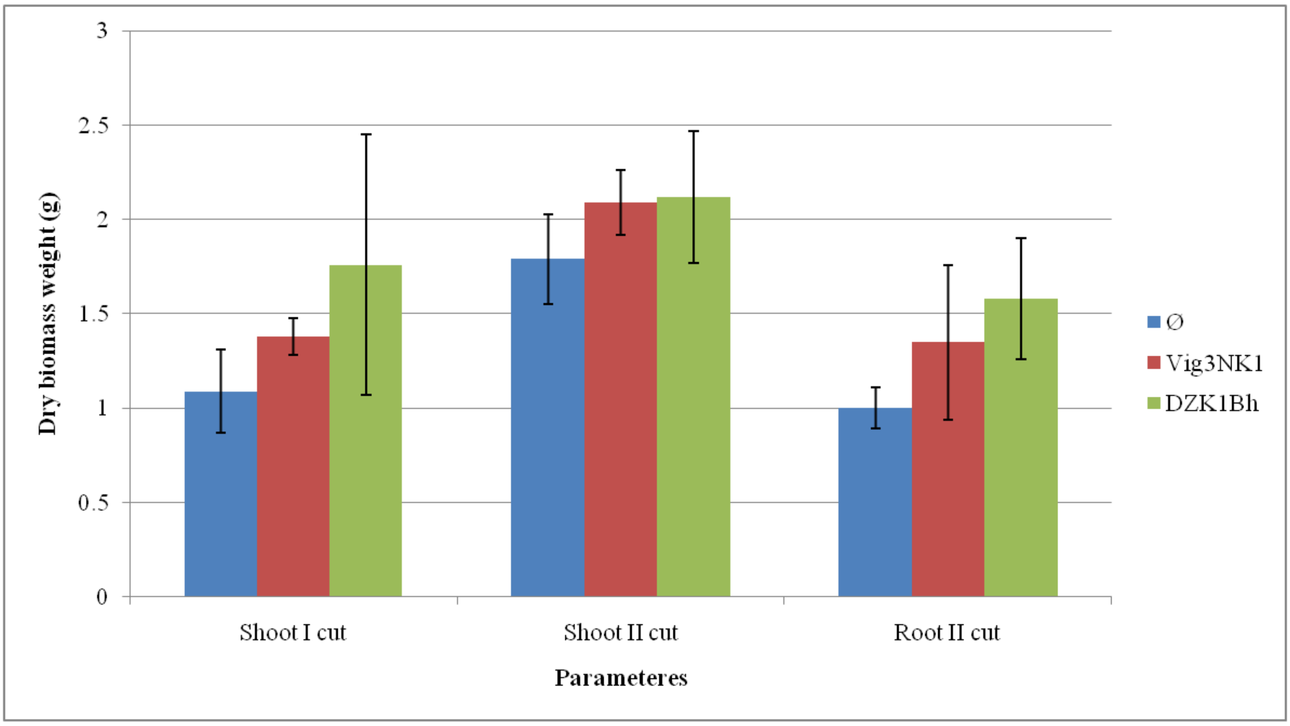

Figure 2. Effect of bacterial inoculation on plant dry weight. Data is presented as average values from three replicates ± standard deviation (SD).

Strain DZK1Bh was previously recommended as an efficient inoculant for improving the growth of orchardgrass in acid soils, where this strain increased the shoot fresh and dry weight (Knežević et al., 2021). Furthermore, the beneficial effects of *B. megaterium* on the growth of different plant species were also previously described by other authors (Bhatt and Maheshwari, 2020; Nascimento et al., 2020; Dahmani et al., 2020).

The results of nitrogen content in the plant material under the influence of bacterial inoculation are shown in Figure 3. Nitrogen content in orchardgrass shoots was slightly increased under the influence of bacterial inoculation in both cuttings. In the first cutting, inoculation by strain DZK1Bh had better effect on the nitrogen content in orchardgrass shoots, while in the second cutting the strain Vig3NK2 showed better results. Regarding the nitrogen content in the root material, values of N% in roots of uninoculated plants had similar values as plants inoculated by strain DZK1Bh. Nitrogen content in plant roots inoculated by Vig3NK2 was slightly lower in comparison to the uninoculated control.

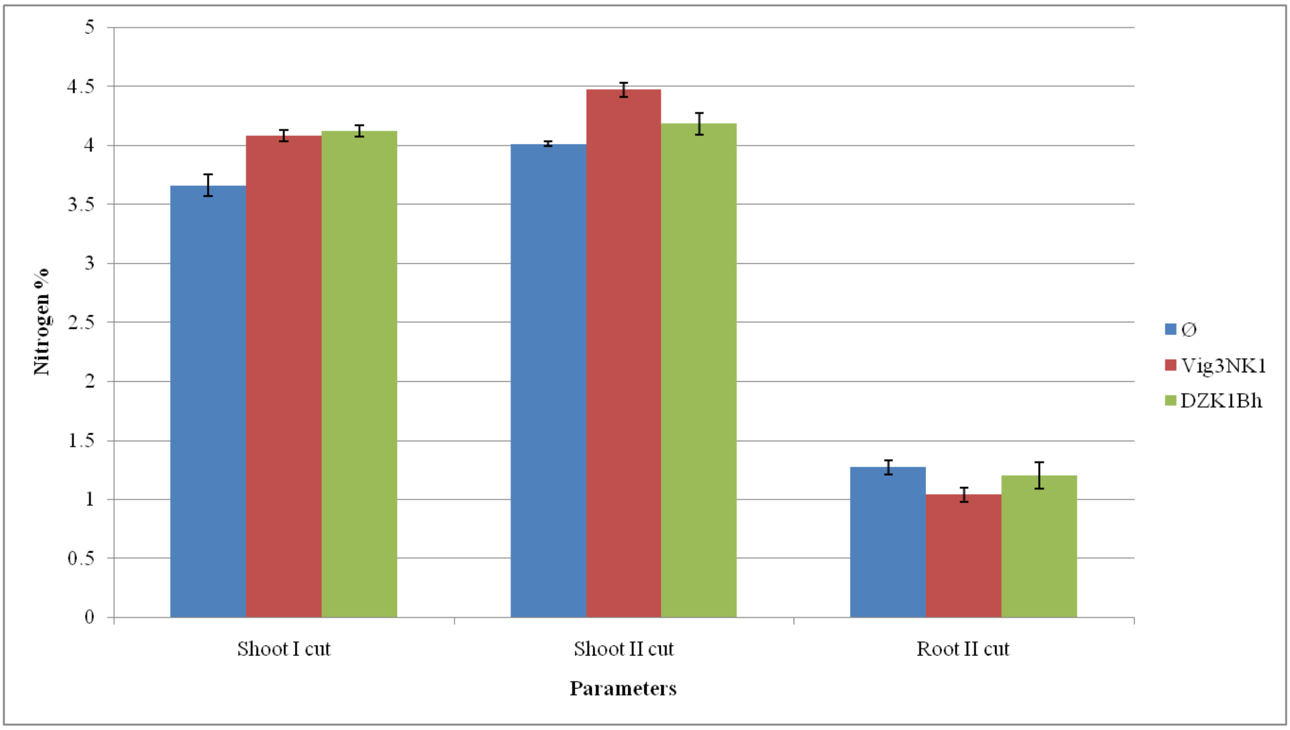

Figure 3. Effect of bacterial inoculation on nitrogen content (N%) in the plant material (shoots and roots). Data is presented as average values from three replicates ± standard deviation (SD).

Overall, the values obtained for N% in the orchardgrass shoot material were higher in comparison to the previously obtained results for the same strain in acid soil, where the highest obtained value for N% was 3.98% (Knežević et al., 2021). Masood et al. (2020) also confirmed the positive effects of inoculation of tomato plant by *B. pumilus*. This could be due to the fact that *Bacillus* species could produce nitrogenase involved in nitrogen fixation and enhance the growth and yield of different plant species (Singh and Shyu, 2024). Processes of atmospheric nitrogen fixation by soil bacteria play important role in supplying soil with nitrogen. Nitrogen fixation can be performed by symbiotic or free-living bacteria. Symbiotic bacteria are associated with legumes, while free-living nitrogen-fixing bacteria are found among different genera. *nifH* gene encoding nitrogenase reductase, among other genetic determinants, is commonly used gene marker for analyzing the ability of bacteria to fix nitrogen (Saxena et al., 2019). In the study by Yousuf et al. (2017), nitrogen fixing ability was observed in different *Bacillus* isolates from tropical estuary and adjacent coastal sea, with *B. megaterium* being most potent. Interesting study about tripartite interaction involving plant, fungus and bacteria concerning N metabolism is done by Paul et al. (2020). Rice inoculated with basidiomycetous fungus *Rhodotorula mucilaginosa* JGTA-S1 which cannot convert nitrate or nitrite to ammonium or fix atmospheric nitrogen, but harbors endobacteria *Pseudomonas stutzeri* with the nitrogen-fixing ability enables the growth of the fungus in N-free conditions and improves growth, nitrogen content and N-use efficiency plants (Paul et al., 2020).

The results of determining the concentration of micro- and macroelements in orchardgrass plants are shown in Tables 3 and 4. In general, concentrations of microelements in both cuts was lower in the plants inoculated by both bacterial isolates, in comparison to the uninoculated control (except in the second cut for Zn and Vig3NK2 inoculation). These results indicate that inoculation of orchardgrass by selected bacterial isolates could reduce the content of microelements in plant material. Similar results regarding the effects of bacterial inoculation on the content of microelements in different species were previously recorded by other authors (Schommer et al., 2023; Wróbel et al., 2023).

Table 3. Concentration of microelements in orchardgrass plants under the influence of bacterial inoculation

Treatment	**As**	**Cd**	**Co**	**Cr**	**Cu**	**Fe**	**Mn**	**Ni**	**Pb**	**Zn**
	mg kg^{-1}									
Ø I cut	BMDL	0.48	0.24	4.61	10.88	447.96	21.10	4.47	0.76	50.77
Ø II cut	BMDL	0.36	0.16	4.00	10.28	304.45	14.00	4.37	0.39	48.33
Vig3NK2 I cut	BMDL	0.42	0.07	3.40	9.27	138.68	20.13	3.74	0.13	46.84
Vig3NK2 II cut	BMDL	0.28	0.11	3.39	10.23	230.74	16.64	3.86	0.28	50.94
DZK1Bh I cut	BMDL	0.38	0.10	3.58	7.36	191.83	20.73	3.62	0.14	39.79
DZK1Bh II cut	BMDL	0.27	0.09	1.75	9.77	204.69	15.35	2.71	0.63	46.15

BMDL – bellow method detection limit

The concentration of Ca in plant material varied depending on bacterial treatment and cutting (Table 4). In plants treated by Vig3NK2 the concentration of Ca was lower in the first cut, and higher in the second, in comparison to the uninoculated control. On the other hand, the concentrations of Ca in plants treated by DZK1Bh were higher in both cuts, in comparison to the uninoculated control. The concentration of K, Mg, and P in plants inoculated by bacterial isolates was higher in comparison to the uninoculated control (except for DZK1Bh treatment in the first cut for K and Mg). Previously it was demonstrated that the effects of bacterial inoculation on the concentration of macro and microelements varies in different cuts in relation to the bacterial treatment, season, location, and plant species (Stajkovic-Srbinovic et al., 2020; Knezevic et al., 2021).

Table 4. Concentration of macroelements in orchardgrass plants under the influence of bacterial inoculation

Treatment	**Ca**	**K**	**Mg**	**P**
	g kg^{-1}			
Ø I cut	9.75	40.14	5.04	2.38
Ø II cut	8.17	33.63	4.77	2.20
Vig3NK2 I cut	8.84	44.45	5.17	2.51
Vig3NK2 II cut	9.52	38.39	4.80	2.63
DZK1Bh I cut	11.36	37.81	4.74	2.22
DZK1Bh II cut	8.59	35.24	4.85	2.30

Bacillus halotolerans Vig3NK2 and *B. megaterium* DZK1Bh have shown good potential as plant growth stimulants under stress conditions caused by increased Ni concentration. They reduced the inhibition percentage of orchardgrass seedling growth by increasing the relative seed germination index. Tested inoculants lowered the concentrations of toxic and potentially toxic metals in orchardgrass biomass and, in general, improved macroelements content in plants cultivated in the pot experiment. The results of this study may help in further field experiments with the phytoremediation of nickel-contaminated soils and production of microbial biofertilizers for contaminated soils.

Acknowledgement

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

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Original paper DOI: 10.5937/ZemBilj2402014B

Poboljšanje prinosa i nutritivnog sastava ježevice u zemljištima sa povećanom koncentracijom nikla inokulacijom *Bacillus* sojevima

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Izvod

Zagađenje zemljišta potencijalno toksičnim elementima u tragovima (PTE) raste iz godine u godinu i postaje ozbiljan problem širom sveta. Rast biljaka je pod uticajem brojnih interakcija sa faktorima sredine i organizmima iz okolnog zemljišta, uključujući i visoke koncentracije TE. Cilj ovog istraživanja bio je procena mogućnosti gajenja ježevice na zemljištima sa povećanom koncentracijom Ni i poboljšanje kvaliteta biljaka u ovim stresnim uslovima primenom *Bacillus* inokulanata. Dodatno, osobine koje promovišu rast, kao i klijavost semena *Dactylis glomerata* inokulisanih *Bacillus* inokulantima su ispitani. Glavni cilj bio je ispitivanje efekata inokulacije na prinos biomase, sastav nutrijenata i koncentraciju mikroelemenata u biomasi ježevice. Sposobnost bakterijskih izolata da tolerišu visoke koncentracije Ni kao i potencijal ovih bakterija da promovišu klijavost semena ježevice testirano je in vitro u Petri šoljama. Ogled u saksijama sproveden je sa zemljištem koje sadrži povišenu koncentraciju Ni i izmerene su suva masa kao i sadržaj mikro i makro elemenata u biljnom materijalu. Rezultati ogleda u saksijama pokazali su pozitivan efekat bakterijske inokulacije na suvu nadzemnu masu ježevice, dok je sadžaj azota u nadzemnom delu bio blago povišen pod uticajem bakterijske inokulacije. Osim toga, sadržaj mikroelemenata u biljnom materijalu bio je redukovan kod inokulisanih biljaka.

Ključne reči: *Bacillus* inokulanti, *Dactylis glomerata,* remedijacija zemljišta, elementi prisutni u tragovima, kvalitet biljne mase

Received 14.10.2024
Revised 12.11.2024
Accepted 18.12.2024