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# Sustainable Forage Crop Production: Application of New *Bacillus* Isolates from Alfalfa Rhizosphere Soil

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**Abstract.** The aim of the present study was to investigate the potential of the new *Bacillus* spp. isolates as promising plant growth promoters in alfalfa cultivation. As one of the most widely grown forage legumes in the world, yield and quality of alfalfa (*Medicago sativa*) are of great importance. A total of five obtained *Bacillus* spp. isolates were tested for plant growth promoting (PGP) traits and applied as single inoculant in alfalfa seed treatments *in vitro*. Isolates I1 and I2 showed all PGP traits (phosphate solubilization and production of indole-3-acetic acid and siderophores). Treatments of alfalfa seeds with five tested isolates induced increase in shoot and root length, compared to the control. The highest shoot and root length of alfalfa seedlings was resulted by seed inoculation with I3 and I1 inoculums, respectively. The results of this study highlight the potential of rhizosphere soil to harbor beneficial bacterial strains that could be exploited for PGP of a broad range of plants.

**Keywords:** *Bacillus* spp.; PGP bacteria; seed germination; seedling shoots and roots; *Medicago sativa*.

## I Introduction

Bio-inoculants, with beneficial soil microorganisms as their active components, are currently considered as an ecologically sound alternative for augmenting soil fertility, crop productivity, and the quality of crops [1,2,3]. The application of bacterial bio-inoculants offers cost-effective alternative and demonstrates potential to replace expensive and harmful agrochemicals including synthetic fungicides [3].

Forage grasslands serve as crucial feed sources for livestock and hold significant economic importance. It is estimated that they cover approximately 70% of the global agricultural area, which accounts for 26% of the total land area [4]. In 2016, the combined production of forage plants, wine, and fruit comprised about one third of the

total crop output within the European Union. The contribution of forage plants to this output was substantial and comparable to that of fruits and wine [5].

Among the commonly cultivated herbaceous legumes [such as trefoil (*Lotus corniculatus*), clover (*Trifolium* spp.), and vetches (*Vicia* spp.)], medics (*Medicago* spp.) represent a significant species. The perennial forage crop alfalfa (*Medicago sativa*) is the most extensively cultivated, as it can be grown in conjunction with both temperate and tropical grasses or as a standalone crop [5].

Therefore, finding effective bacterial strains to be used as active agents of bio-inoculants for improving the growth and productivity of alfalfa is highly important. Bio-inoculants based on *Bacillus* spp. show great potential for enhancing agricultural plant growth and yield through their ability to improve nutrient availability, suppress pathogens, and mitigate abiotic stress.

The aim of this research was to isolate *Bacillus* spp. from rhizosphere soil of alfalfa, to evaluate their PGP (plant growth promoting) characteristics, and to validate their beneficial effect on improving alfalfa seed germination *in vitro*.

## II Materials and methods

### 2.1. Isolation of Rhizobacteria

Isolation of the plant growth promoting (PGP) bacteria was performed from the rhizosphere soil of alfalfa (soil with neutral pH value of 6.8 and percentage of soil organic matter - SOM of 3.12%). Ten g of soil (fresh weight) was dissolved in 100 mL of sterilized distilled water and shaken for 15 min on a rotary shaker to obtain a soil extract. Soil extract (various dilutions) was then heated at 80°C for 15 min to obtain *Bacillus* spp. endospores, spread on the nutrient agar (NA) plates (0.5 mL), and incubated at 28°C for 48 h.

Pure bacterial cultures were obtained by transferring all *Bacillus*-like colonies, exhibiting different morphology on new NA plates. Plates were incubated at 28°C for 24 h and the isolates were stored at 4°C until the moment of use. The isolates were stored at 4°C on inclined NA.

### 2.2. Indole-3-Acetic Acid (IAA)

The ability of bacterial isolates to produce IAA was determined by using nutrient broth (NB) with and without tryptophan (2 mg $mL^{-1}$) enrichment, following the procedure previously described by [6]. The concentration of IAA (development of a pink colour by adding Salkowski reagent, 0.01 mol $L^{-1}$ of $FeCl_3$ in 35% $HClO_4$) was measured by spectrophotometer at 530 nm, after 25 min of incubation in the dark.

The test was performed in three replicates for each isolate. The production of IAA was determined based on the comparison to the standard curve of IAA.

### 2.3. Phosphate Solubilization

The ability of bacterial isolates to solubilize inorganic phosphates was tested using Pikovskaya medium (PVK) by the procedure described in Rokhbakhsh-Zamin [7].

The appearance of halo zone around the colony after 7 days of incubation at 28°C was considered as a positive result.

### 2.4. Siderophore Production

Siderophore production was determined by Chrome Azurol S (CAS) Blue agar plate assay, according to procedure described by Milagres (1999) [8]. Bacterial cultures were spot inoculated onto plates and incubated (28°C, 5 days) in the dark.

The colony surrounded by the orange halo zone (measured in mm) was considered to produce siderophores.

### 2.5. Seed Germination in *Vitro*

The ability of bacterial isolates to induce germination of alfalfa seeds was accessed *in vitro* on Petri dishes by using filter paper method. Twenty seeds of alfalfa were soaked in over-night bacterial culture for each isolate (treatments I1-I5), while non-inoculated seeds were used as the control sample (control treatment). Seeds were then air-dried at room temperature (22-24°C), and Petri dishes were kept for two weeks in a transparent sealed box.

Final seed germination percentage (FG%) was estimated in accordance with the methodology outlined by Ali et al. (2015) [9], while the length of alfalfa seedling shoots and roots was expressed in cm. All tests were done in three independent replications.

### 2.6. Statistical Analysis

Results for seed germination tests and the evaluation of effects of bacterial inoculation of alfalfa early development parameters were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's test.

## III Results and Discussion

A total of five *Bacillus*-like isolates was isolated from the selected alfalfa rhizospheric soil. The results of the qualitative and quantitative tests for the production of IAA, siderophores and for phosphate solubilization by the five isolates are presented in the Table 1. Isolates I1 and I3 had the ability to produce IAA without and with presence of 2 µg $mL^{-1}$ tryptophan in the growing medium. On the other hand, isolates I2 and I5 produced IAA only in presence of tryptophan. In the phosphate solubilization test, halo zone was not detected only for isolate I3, while other isolates had the ability to solubilize phosphates. All tested isolates were able to produce siderophores and the largest orange halo zone was produced by I1 (11.17±0.58 cm).

**Table 1**. Plant growth promoting traits of bacterial isolates

| Isolate | IAA (µg $mL^{-1}$) | | Phosphate solubilization | Siderophore (mm) |
|---|---|---|---|---|
| | $TRP_0$ | $TRP_2$ | | |
| **I1** | 3.78±0.13 | 11.11±0.16 | + | 11.17±0.58 |
| **I2** | - | 4.81±0.14 | + | 7.17±0.76 |
| **I3** | 4.74±0.17 | 10.30±0.25 | - | 6.33±0.76 |
| **I4** | - | - | + | 4.33±0.58 |
| **I5** | - | 1.04±0.06 | + | 4.33±0.29 |

The synthesis of indole-3-acetic acid by plant growth promoting bacteria, particularly within the *Bacillus* genus, is closely associated with beneficial effects on plant development. Bacterial IAA has the potential to significantly impact plant growth by influencing key processes including the ones related to the germination and early development stage [10]. In addition, phosphate-solubilizing bacteria possess the capacity to enhance crop yields and uphold environmental sustainability by rendering insoluble phosphate accessible to plants through solubilization and mineralization processes [11]. Besides their effect in plant growth promotion, siderophores producers can rescue iron by converting inorganic to organic forms in the rhizosphere making it unavailable for pathogens [12]. Previous studies by various authors have also identified siderophores production as one of biocontrol mechanism against fungal plant pathogens [13, 12, 14]. *Bacillus velezensis* and *B. paramycoides* were characterized as siderophore producers, as well as effective biocontrol agents [13].

The results regarding the observed effect of the six tested bacterial isolates on seed germination of alfalfa are presented in Figure 1. Isolates I1 and I5 induced final germination percentage (FG%) of alfalfa seeds of 100%. However, values of FG for all bacterial treatments were not statistically different from the control (96.67%). Values of final seed germination for other tested isolates ranged between 90% (I4) and 96.67% (I3).

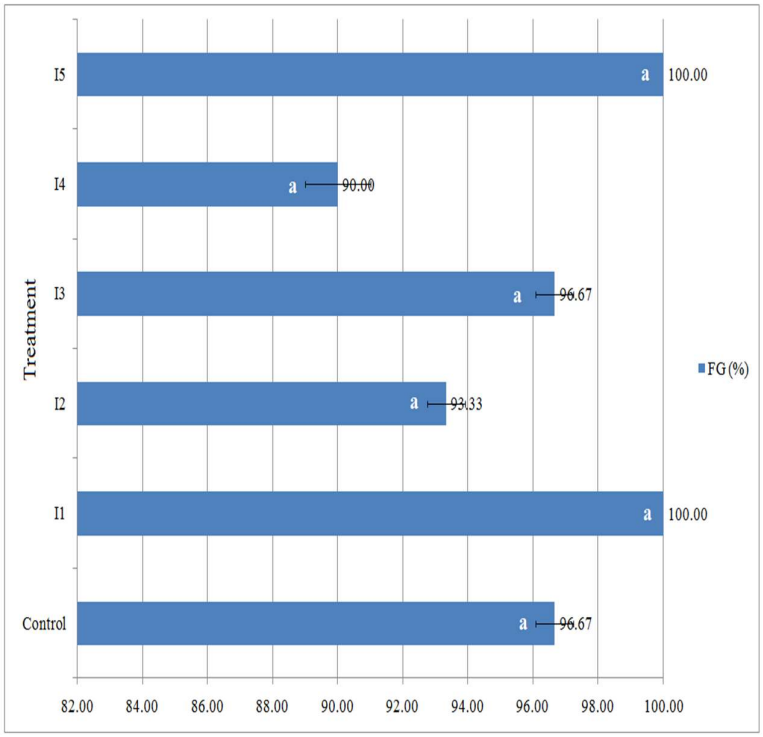


**Figure 1.** Final seed germination (FG%) of five tested isolates and non-inoculated control

Bacterial inoculation had different effect on shoots and roots length of alfalfa seedlings (Figure 2). For shoots of alfalfa seedlings, only treatments with I5 had values which were statistically higher than uninoculated control. Treatment with other tested isolates showed values of shoot lengths that were not statically different form the non-inoculated control. Values for roots of alfalfa seedlings treated with isolates I1, I3 and I5 did not differ from the non-inoculated control, while isolate I1 stood out with the highest values.

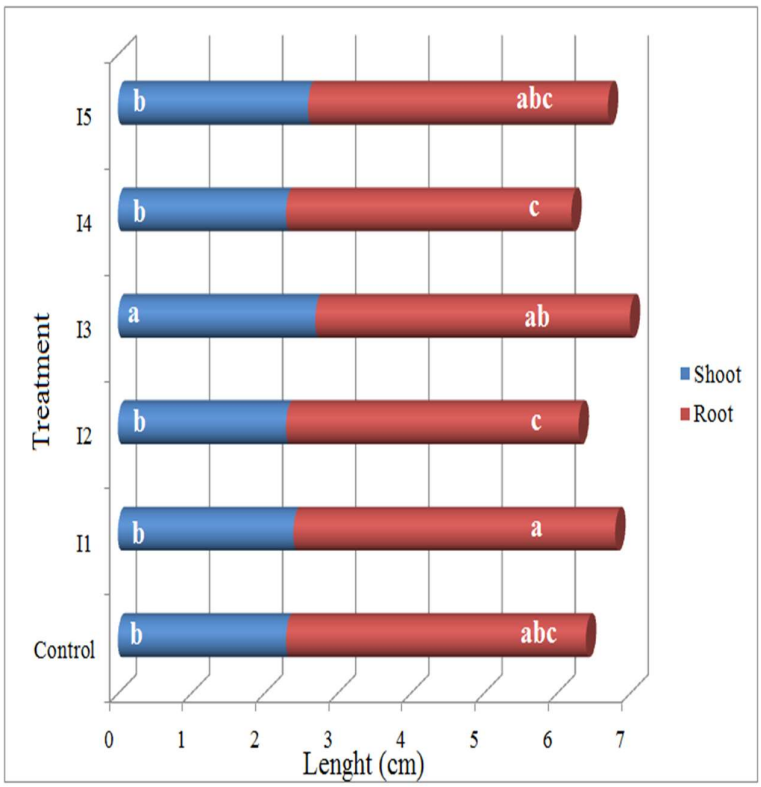


**Figure 2.** Effect of bacterial inoculation on shoots and roots (cm) of alfalfa seedlings

Beneficial effects of bacterial inoculation on alfalfa seed germination has been previously recorded [15]. Similarly, application of bacteria increased the vigor index of seedling, percentage and germination rate, but did not affect other alfalfa traits under laboratory conditions, *in vitro* [16]. Further, combination of bacterial consortium alone and with fertilizers improved alfalfa growth under stress environment of oily sludge contamination soils [17].

In general, bacterial isolates applied in this research showed beneficial effect on alfalfa seed germination and on shoot and root lengths as early growth parameters (Figure 3).

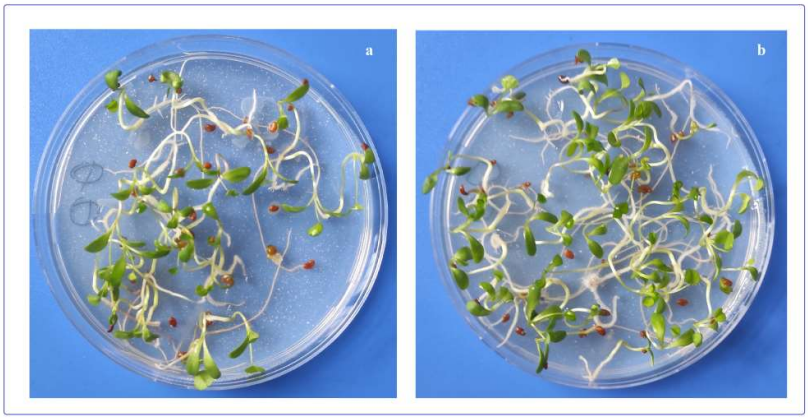


**Figure 3.** Effect of bacterial inoculation on alfalfa seedling (a: non-inoculated control, b: bacterial inoculation)

In conclusion, this research demonstrated that bacterial isolates with PGP traits not only enhance seed germination percentage but also improve alfalfa seedling growth parameters, corroborating previous studies that indicated the potential of *Bacillus* spp. in promoting plant health and productivity. This indicates that harnessing *Bacillus* spp. with PGP characteristics holds significant promise for improving alfalfa seed germination and seedling development in the context of general alfalfa production. Further research and practical application are essential to fully realize the potential of these beneficial bacteria in enhancing crop yields and promoting sustainable agriculture.

## References

1. Lengai, G.M.W., Muthomi, J.W. “Biopesticides and their role in sustainable agricultural production. Journal of Biosciences”. 6(06), 7 (2018).
2. Suthar, H., Hingurao, K., Vaghashiya, J., Parmar, J. “Fermentation: a process for biofertilizer production”. In: Panpatte, D.G., Jhala, Y.K., Vyas, R.V., Shelat, H.N. (eds.) Microorganisms for Green Revolution, pp. 229-252. Springer, Singapore. (2017).
3. Singh, J.S., Koushal, S., Kumar, A., Vimal, S.R., Gupta, V.K. “Microbial Inoculants in Sustainable Agricultural Productivity, Vol. II: Functional Application”. Springer, New Delhi (2016).
4. FAO., Grassland Index. A searchable catalogue of grassland forage legumes. Rome, Italy: FAO (2014).
5. Capstaff, N.M., Miller, A.J. “Improving the yield and nutritional quality of forage crops. Frontiers in Plant Science”. 9, 535 (2018).
6. Gordon, S.A. Weber, R.P. “Colorimetric Estimation of Indoleacetic Acid. Plant Physiology”. 26, 192-195 (1951).
7. Rokhbakhsh-Zamin, F., Sachdev, D., Kazemi-Pour, N., Engineer, A., Pardesi, K.R., Zinjarde, S., Dhakephalkar, P.K. Chopade, B.A., “Characterization of plant-growth-promoting traits of Acinetobacter species isolated from rhizosphere of Pennisetum glaucum. Journal of Microbiology and Biotechnology”. 21(6), 556-566 (2011).
8. Milagres, A.M., Machuca, A., Napoleão, D. “Detection of siderophore production from several fungi and bacteria by a modification of chrome azurol S (CAS) agar plate assay. Journal of Microbiological Methods”. 37(1), 1-6 (1999).
9. Ali, A., Phull, A.R., Zia, M., Shah, A.M.A., Malik, R.N. “Phytotoxicity of River Chenab sediments: In vitro morphological and biochemical response of Brassica napus L. Environmental Nanotechnology, Monitoring and Management”. 4, 74-84 (2015).

10. Figueredo, E. F., Da Cruz, T. A., De Almeida, J. R., Batista, B. D., Marcon, J., De Andrade, P. a. M., De Almeida Hayashibara, C. A., Rosa, M. S., De Azevedo, J. L., Quecine, M. C. "The key role of indole-3-acetic acid biosynthesis by *Bacillus thuringiensis* RZ2MS9 in promoting maize growth revealed by the ipdC gene knockout mediated by the CRISPR-Cas9 system. Microbiological Research". 266, 127218 (2023).
11. Cheng, Y., Narayanan, M., Shi, X., Chen, X., Li, Z., Ma, Y. "Phosphate-solubilizing bacteria: Their agroecological function and optimistic application for enhancing agro-productivity. Science of the Total Environment". 166468 (2023).
12. Khalil, M. M. R., Fierro-Coronado, R. A., Peñuelas-Rubio, O., Villa-Lerma, A. G., Plascencia-Jatomea, R., Félix-Gastélum, R., Maldonado-Mendoza, I. E. "Rhizospheric bacteria as potential biocontrol agents against Fusarium wilt and crown and root rot diseases in tomato. Saudi Journal of Biological Sciences". 28(12), 7460 (2021).
13. El-Sersawy, M.M., Hassan, S.E.D., El-Ghamry, A.A., El-Gwad, A.M.A., Fouda, A. "Implication of plant growth-promoting rhizobacteria of *Bacillus* spp. as biocontrol agents against wilt disease caused by *Fusarium oxysporum* Schlecht. in *Vicia faba*. L. Biomolecular Concepts". 12(1), 197-214 (2021).
14. Gowtham, H.G., Hariprasad, P., Nayak, S.C., Niranjana, S R."Appl ication of rhizobacteria antagonistic to *Fusarium oxysporum* f. sp. *lycopersici* for the management of Fusarium wilt in tomato. Rhizosphere". 2, 72-74 (2016).
15. Knežević, M., Stajković-Srbinović, O., Assel, M., Milić, M., Mihajlovski, K., Delić, D. Buntić, A. "The ability of a new strain of *Bacillus pseudomycoides* to improve the germination of alfalfa seeds in the presence of fungal infection or chromium. Rhizosphere". 18, 100353 (2021).
16. Alikooyi, M.S., Sourki, A.A., Kiyani, S., Dehkordi, M.M. "Effect of plant growth promoting bacteria on Seed germination and seedling emergence of alfalfa (Medicago sativa L.) hamedani ecotype in the laboratory, morphological characteristics and pigment contents in greenhouse conditions. Iranian Journal of Seed Science and Technology". 4, 173-187 (2020).
17. Shahzad, A., Bano, A., & Siddiqui, S. "Effect of bacterial consortium on alfalfa (*Medicago sativa* L.) plant nutrient uptake and antioxidant enzymes at different levels of oily sludge. Israel Journal of Plant Sciences". 67(3–4), 158–170 (2020).