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Effect of Co-Immobilized Tri-Bacteria into Alginate Beads on Growth and Root Mycorrhizal Colonization Potential of *Medicago Sativa* Plants

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Abstract: To develop an inoculant, the potential of three plant growth-promoting rhizobacteria (PGPR) isolated from southern Morocco, namely *Pseudomonas putida* strain NTM22, *Klebsiella* sp. strain NTCC1, and *Pseudomonas cedrina* subsp. *Fulgida strain* NTCC12 was assessed. At first, these PGPR were tested for their phosphate (P) solubilization capacity in the free state and in the immobilized state in Na-alginate beads. The immobilized bacteria NTM22, NTCC1, and NTCC12 recorded a mean maximum concentration of soluble P of 171.18, 151.62, and 181.62 µg/mL, respectively, while the mean maximum concentrations recorded by free bacteria were 92, 135.3, and 126.3 µg/mL, respectively. Investigation of the effect of the consortium (NTM22, NTCC1, and NTCC12) showed a P solubilization of 160.6 µg/mL by free cells and 189.6 µg/mL by encapsulated cells. Subsequently, the potential of using the consortium as a bioinoculant for alfalfa plants was assessed under greenhouse conditions. The overall results showed that the bacterial consortium in the free and immobilized state had improved significantly ($p \leq 0.05$) the shoot dry weight of the plants by 34.7% and 52% and root dry weight by 50% and 61%, respectively, relative to the uninoculated control plants. It also increased significantly ($p \leq 0.05$) the frequency (F%) of arbuscular mycorrhization (AM) by 28.4% and 22%, the intensity of mycorrhization (M%) by 44.8% and 44.4%, and the richness in arbuscules (A%) by 61% and 52%, respectively, compared to the control plants. This study proved that the encapsulated forms of bacteria, especially the consortium, have an interesting potential to be developed as a bioinoculant to improve plant growth and interaction with AM fungi which should be important in sustainable agriculture.

Keywords: plant growth promoting rhizobacteria; *Medicago sativa*; bioinoculant; arbuscular mycorrhiza; alginate beads.

1. Introduction

Phosphorus is the second most important nutrient for the growth and development of plants. In nature, the maximum amount of phosphorus is concentrated in the surface part of the soil, in insoluble mineral form from weathering rocks (rock phosphate, tricalcium phosphate, dicalcium phosphate, hydroxyapatite) or organic compounds resulting from the degradation of plants by soil flora and fauna. The deficiencies of inorganic phosphate (orthophosphate) in soils

seriously limit growth and crop productivity in most regions of the globe [1-5]. Consequently, in most agroecosystems, P enrichment of the soil by P fertilizer applications is considered important for agronomic crop production levels. However, over 70-90% of the applied phosphate fertilizers bind to the soil, making them unavailable for plant uptake and causing unanticipated environmental impacts [6,7]. Most of the phosphorus added in soluble form is either adsorbed in alkaline soils by the calcium present on the exchange complex or precipitated in acidic soils by the free forms of iron or aluminum, which is found in significant quantities in soils [8].

The concept of converting unavailable inorganic phosphate into available forms H_2PO^- and HPO^{2-} for uptake by the plant is a phenomenon named solubilization of mineral phosphate. This phenomenon is facilitated by certain microorganisms such as fungi, actinomycetes, arbuscular mycorrhizae (AM), and bacteria like phosphate-solubilizing bacteria (PSB) [1,9-13]

Some bacteria, plant-growth-promoting rhizobacteria (PGPR), in addition to the solubilization of minerals (P and K), are able to improve plant growth thanks to several characteristics such as nitrogen fixation, synthesis of auxin, cytokinin, ethylene, v$itamins, and other important plant's hormones essential for enhancing growth and/or attenuating the harmful effect of pathogens [14-17]. These bacteria can be used in agriculture by managing native populations or inoculating selected strains with a higher capacity [5,18]

PGPR can also interact with arbuscular mycorrhizal fungi (AMF) and plant tissues. This cooperation is beneficial to plant growth due to augmented nutrition, bacterial survival, protection against biotic and abiotic stresses, and plant root hyphal permeability [19-21]. PGPR can enhance the establishment of AMF through different mechanisms, such as mycelial growth, root colonization, and stimulating spore germination [22]. On the other hand, AMF improves the ability of rhizospheric bacteria to perform various functions adequately. For instance, AMF enhances the activity of phosphorus-solubilizing and nitrogen-fixing bacteria and consequently promotes plant growth [13,23]. They also increase effective phosphatase secretion to the rhizosphere by regulating bacteria's protein secretory systems, which enhances the phosphate transfer process in AMF hyphae [24].

Inoculants should be developed as products with longer shelf-life stability. The requirements for their shelf life vary from 2-3 months to 1-2 years at room temperature. Maximizing the initial quantity of viable cells in inoculants is a strategy to compensate for the rate of rapid deterioration [25,26]. Therefore, the inoculant formulations aim to allow higher survival of PGPR during storage and to be at the site of application in suitable and available forms. This could be ensured by encapsulating the cells in biodegradable capsules [27]. For example, the encapsulation of rhizobacteria can enhance cell survival during storage, and encapsulated cells could be released into the target medium in a slow and controllable manner, increasing long-term effectiveness [28]. Several studies have commonly used alginate gel as the primary ingredient for microcapsules for bacterial immobilization because of its biodegradability and environmental sustainability compatibility [29,32-34].

Several authors have stated that PGPR species increase plant growth and yield in free and immobilized states, single or in consortium with AMF [29,35-37]. However, there are no findings on the impact of the PGPR consortium immobilized in alginate beads on alfalfa plant growth and AM root colonization with native AMF. Therefore, a suitable formulation, encapsulating PGPR with sodium alginate, to enhance growth and AMF colonization will be important and effective in sustainable agriculture.

In this study, we investigated the phosphate solubilizing ability of free and encapsulated *Pseudomonas putida* strain NTM22, *Klebsiella* sp. strain NTCC1, and *Pseudomonas cedrina* subsp. Fulgida strain NTCC12 and their consortium and their effects on alfalfa growth and root AM colonization by indigenous AMF.

2. Materials and Methods

2.1. Bacterial strains.

The bacterial strains used in this work were *P. putida* strain NTM22, Klebsiella sp. strain NTCC1, and *P. cedrina* subsp. *Fulgida* strain NTCC12 was previously isolated from southern Morocco [38]. They have been shown to be effective as PGPR has many interesting PGP traits, such as N2 fixation, P solubilization, ammonia, lytic enzymes, siderophores production, etc. [38].

2.2. Bacterial encapsulation of cells in sodium alginate

Alginate beads preparation was carried out according to Bashan [39] method with some modifications. Bacteria were cultured in 500 mL Erlenmeyer flasks containing 250 mL Lauria Bertani (LB) Broth. After 24 hours of incubation at 28 °C on a rotary shaker (150 rpm), cells were collected by centrifugation (6000 g for 10 minutes) in 50 mL tubes. Then, after removing the supernatant, the bacterial pellet was washed twice with sterile distilled water and then suspended in 10 mL of sterile distilled water. The optical density OD600 nm was set to 0.4, which equates to approximately 10^8 CFU mL^{-1}. This suspension was used to prepare the isolates individually or in a consortium immobilized in alginate beads.

Sodium alginate and bacterial suspension were mixed well (v, v) with gentle stirring for 5 min using a magnetic bar to obtain a bacterial concentration of 1 x 10^8 CFU mL^{-1}. The mixture was introduced into a sterile 5 mL syringe to make beads in the sterile solution of $CaCl_2$ 0.1 M.

The beads immersed in the $CaCl_2$ solution were stirred for 15 min and recovered after 3 washes with sterile distilled water to remove debris and unpolymerized cells and kept in the $CaCl_2$ solution until usage (P. solubilization in NBRIP-medium and inoculation of plants).

2.3. Tricalcium phosphate solubilization by immobilized cells.

Tricalcium phosphate solubilization experiments were performed in 250 mL Erlenmeyer flasks containing 100 mL of sterile NBRIP medium (10 $g.L^{-1}$ D-glucose, 5 $g.L^{-1}$ $Ca_3(PO_4)$, 5 $MgCl_2$ $6H_2O$, 0.25 $g.L^{-1}$ $MgSO_4$ H_2O, 0.2 $g.L^{-1}$ KCl, 0.1 $g.L^{-1}$ $(NH_4)_2SO_4$, 15 $g.L^{-1}$ agar, pH 7) and inoculated with free and immobilized cells by immersing 3 g of bacterial beads. Then, the Erlenmeyer flasks were incubated at 28 °C with continuous shaking at 120 rpm. Soluble phosphate was measured every day for 5 days. Soluble phosphate was analyzed according to the technique described by Fiske and Subbarow [40] with some modifications. 2 mL of the samples were centrifuged (10,000 g for 10 min) and filtered using a 0.22 µm Millipore® filter. The supernatant was used for the determination of soluble phosphate. 500 µL of the supernatant were mixed with trichloroacetic acid (5.5%). After 10 min of incubation, 500 µL of a coloring reagent (40 mL of ammonium molybdate (1.5%) in sulfuric acid (5.5%) with 10 mL of iron sulfate (2.7%) in distilled water) was added. The phosphomolybdate

produced was measured spectrophotometrically at 700 nm. The calibration curve was prepared with KH_2PO_4 at concentrations between 0.6 and 40 mg/L.

2.4. Pot experiment.

Experiments were carried out in plastic pots with soil (250 g per pot) obtained from agricultural land in the Fez area (33°56'N, 5°13'W, 499 m altitude). The soil of the experiments has the following characteristics: pH 8.1, organic matter 12.93 g/Kg, available N 0.73 g/Kg, and available phosphate 13.25 mg/Kg. In addition, the amount of AMF propagules in the used soil was checked (mycelium, spores, and infected roots). As a result, the indigenous population of soil AMF was used to reflect the AMF inoculum.

Alfalfa (*Medicago sativa*) seeds were surface-sterilized by soaking in ethanol (70%) for 1 min followed by sodium hypochlorite (5.25%) for 5 min, and then washed in sterile distilled water. The seeds were germinated in water agar (7 g/L) for two days in the dark at 28 °C. Plantlets were then transplanted to the culture pots, with 3 plants per pot (3 pots for each treatment). The pots were inoculated with the bacteria (*P. putida* NTM22, *Klebsiella* sp. NTCC1, and *P. cedrina* subsp. *Fulgida* NTCC12) in a free state (suspension of each bacterial culture) and immobilized in Na-alginate beads (10^8 CFU mL^{-1}). Saline solution and sterilized alginate beads were added to the uninoculated plants. Pots were positioned in a greenhouse (approximately 16 h photoperiod, 26–30 °C day and 18-22 °C night) and watered regularly. Five weeks later, plants were harvested and washed with deionized water, and then divided into roots and shoots and dried at 65 °C for 72 h to obtain dry weights.

2.5. AM colonization.

Evaluation of AM colonization of roots was carried out according to Phillips and Hayman's [41] method. Roots were digested in potassium hydroxide (KOH) (10%) at 90 °C for 45 min and then stained with trypan blue (0.5 g/L) in acid glycerol at 90 °C for 15 min. 30 root fragments from each plant were randomly chosen and placed in glycerin on slides. The percentage of colonization of roots was calculated using Trouvelot *et al*. [42] method.

2.6. Statistical analysis.

The data collected were analyzed in one-way ANOVA to determine the significance of the values using the Tukey test. The analyses were realized using Minitab 18 software, and statistical significance was considered at $P \leq 0.05$.

3. Results and Discussion

3.1. Kinetics of solubilization of tricalcium phosphate in vitro by free and immobilized cells in alginate beads

The encapsulated bacteria NTM22, NTCC1, and NTCC12 recorded a mean soluble P concentration after 5 days of 171.18, 151.62, and 181.62 µg/mL, respectively, while the mean concentrations recorded by free bacteria were 92, 135.3, and 126.3 µg/mL, respectively (Fig. 1). The consortium (NTM22, NTCC1, and NTCC12) showed a P solubilization of 160.6 µg/mL by free cells and 189.6 µg/mL by encapsulated cells.

Thus, the immobilized bacteria NTM22, NTCC1, and NTCC12 and their consortium showed better solubilization of tricalcium phosphate compared to free cells. Other works have

reported similar results with microorganisms, such as Aspergillus awamori [43], *Bacillus subtilis* [44], *Serratia marcescens* CTM 50650, and *Enterobacter* sp. US468 [29], *Bacillus velezensis* [34,45]. The results presented here demonstrate that with a gel-cell system, the amount of soluble P can be significantly increased compared to that obtained by cells in free suspension. Therefore, the encapsulated cells can be said to retain their biological functions with increased stability, which can often lead to increased or improved cellular activity [46].

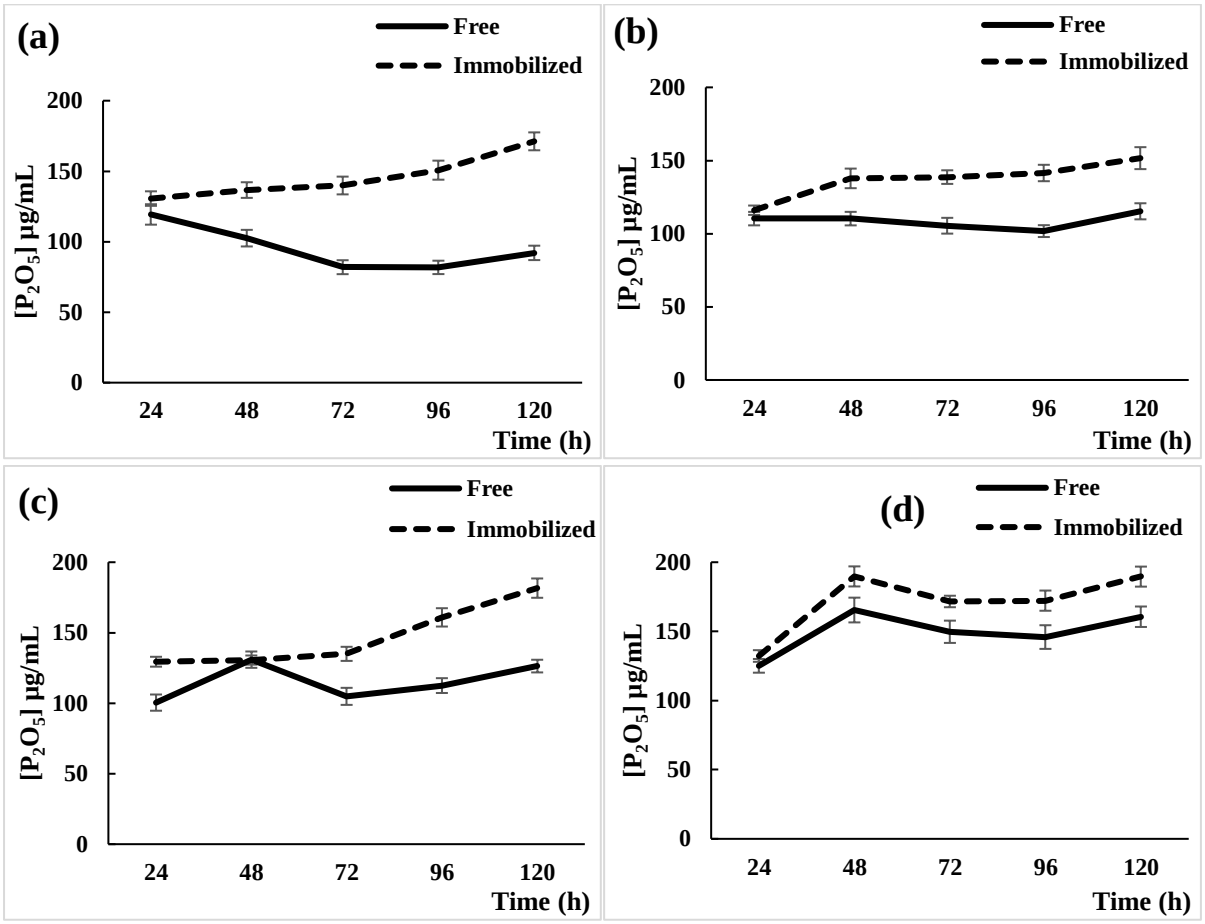

Figure 1. Kinetics of tricalcium phosphate solubilization in NBRIP-medium by the bacterial isolates **(a)** NTM22**, (b)** NTCC1, **(c)** NTCC12, and **(d)** their consortium in the free and immobilized state in the Na-alginate beads. Values are means ± SE.

3.2. Effect of immobilized bacteria on the growth of M. sativa.

The results showed that plant inoculation by the consortium in the free and immobilized state significantly increased ($p < 0.05$) the shoot dry weight of the plants by 34.7% and 52%, respectively, and root dry weight by 50% and 61%, respectively, relative to the uninoculated control plants (figure 2).

Similar results were observed by Farhat *et al*. [29], who concluded that the encapsulation of two phosphate-solubilizing bacteria *S. marcescens* and *Enterobacter* sp. had a stimulating effect on the growth of corn plants. Trivedi and Pandey [15] also reported that *B. subtilis* and *P. corrugata* encapsulated in alginate beads stimulate wheat plants. Recently, Saberi-Rise and Moradi-Pour [31] showed a positive effect of nano-encapsulated *B. subtilis* Vru1 prepared with sodium alginate on the dry weight of the shoot and root parts of bean plants.

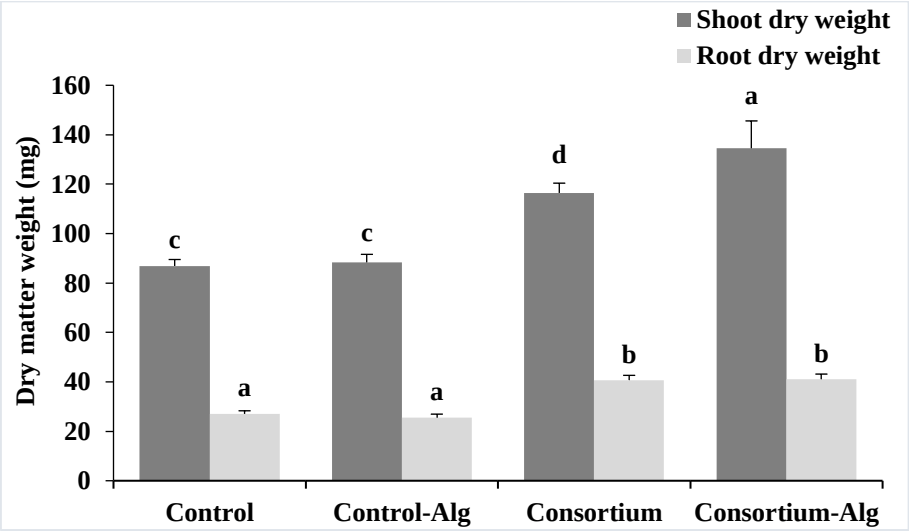

Figure 2. Effect of bacterial consortium (bacterial isolates NTM22, NTCC1, and NTCC12) free and immobilized state in Na-alginate beads on the growth of *M. sativa* plants. Values are means ± SE. Values indexed with the same letter are not significantly different between treatments ($p<0.05$).

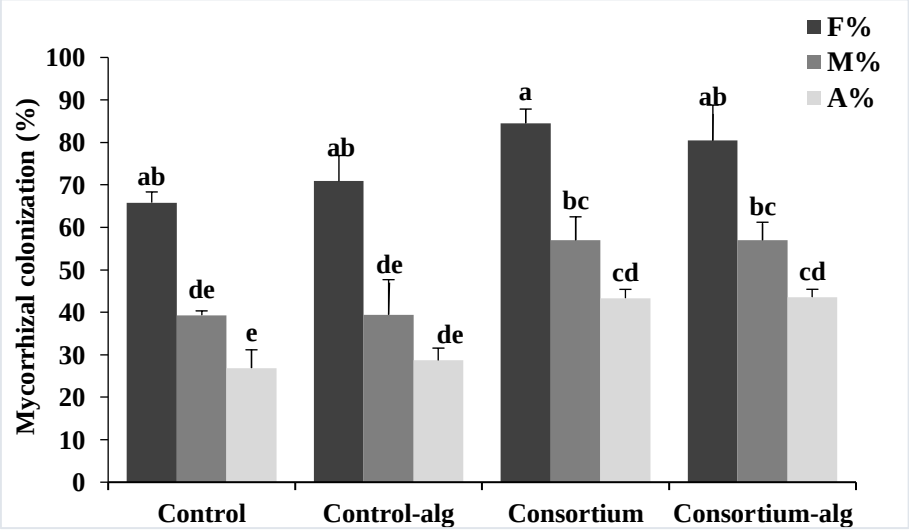

Figure 3. Effect of the bacterial consortium (NTM22, NTCC1, and NTCC12) in the free and immobilized state in Na-alginate beads on the root's arbuscular mycorrhizal (AM) colonization (F%: The frequency of mycorrhization, M%: The intensity of mycorrhization, A%: The richness in arbuscules) of *M. sativa* plants. Values are means ± SE. Values indexed with the same letter are not significantly different between treatments ($p<0.05$).

Microbial encapsulation in alginate beads provides an environment that helps to protect cells from biotic and abiotic stresses and enhances the survival of the bacterial cells. PGPR aids plant growth via multiple mechanisms, which include improved plant physiology and tolerance to various phytopathogens through a variety of actions [47]. These involve nutrient uptake, biotic and abiotic stress neutralization, and disease prevention by volatile organic compounds (VOC) and enzyme secretion. However, PGPR may use different types of mechanisms of action depending on the host plant [48], biotic factors (stages of development, plant genotypes, plant defense mechanisms, interaction with indigenous microbial community), and abiotic factors (such as soil composition, and climatic conditions) [49]. Therefore, the positive effects of our isolates on plant growth would be linked to their PGP traits like nitrogen fixation, phosphate solubilization, IAA, siderophores, lytic enzymes secretion etc.

3.3. Effect of immobilized bacteria on AM colonization.

Inoculation of plants by the bacterial consortium (NTM22, NTCC1, and NTCC12) in the free and immobilized state has a positive effect on the mycorrhizal colonization (F%, M%, and A%) of M. Sativa roots (Figure 3). The frequency of mycorrhization (F%) increased by 28.4% and 22%, the intensity of mycorrhization (M%) increased significantly ($p < 0.05$) by 44.8% and 44.4%, and the richness in arbuscules (A%) by 61% and 52% by the bacterial consortium (NTM22, NTCC1, and NTCC12), in the free state and immobilized in alginate beads, respectively, compared to the uninoculated control plants. We noticed in this experiment that the immobilization of bacteria in alginate beads did not cause any significant negative effect on mycorrhizal colonization. This proves that the Na-alginate beads immobilization retained the physiological activities of the bacterial cells. In fact, alginate beads can protect microorganisms from biotic and abiotic stresses and improve persistence, physiological activity, and cell density [50]. Trapping also allows easy delivery of microbial inoculants to the site where they are needed and can be used in planters commonly used by farmers [51].

Alginate entrapment has been reported in many studies of a single isolated microorganism (e.g., AMF [52] or PGPR [30]), while much fewer studies have been reported on consortia. For instance, De Jaeger *et al*. [53] and Buysens *et al*. [54] co-trapped AMF *R. irregularis* MUCL 41833 with the fungus *Trichoderma harzianum* MUCL 29707 in alginate beads for field trials, Seenivasagan and Babolala [55] studied the utilization of microbial consortia as biofertilizers and biopesticides for the production of feasible agricultural product, the results showed a remarkable increase in potato yield. Vassilev *et al*. [56] also found that the yeast culture encapsulated in the alginate beads behaved as a "mycorrhizal helper microorganism", enhancing the mycorrhization of tomato roots. Loján et al. [37] recently studied the effect of co-trapping *P. plecoglossicida* R - 67094 and *R. irregularis* MUCL 41833 in alginate beads and found an improvement in the colonization of potato seedling roots by AMF.

To our knowledge, there is little work that focuses on the study of PGPR encapsulated in alginate beads on AM colonization. This study demonstrates the beneficial effect of PGPR encapsulated in alginate beads on the mycorrhizal colonization of the roots of alfalfa plants.

4. Conclusions

We conclude that the encapsulation of bacteria (*P. putida* strain NTM22, *Klebsiella* sp. strain NTCC1 and *P. cedrina* subsp. *Fulgida* strain NTCC12) in alginate beads exhibits many advantages. It could help to maintain the physiological activity of bacteria and, thus, a high survival rate during storage. An increased P solubilization rate, as well as a synergy effect between the different strains studied in the present work, was observed. Moreover, encapsulated bacteria stimulate growth and root colonization by indigenous AMF of alfalfa species. All these results show that encapsulated bacteria into alginate beads have a great potential to be developed as a bio-inoculation method that could be beneficial in sustainable agriculture.

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Conflicts of Interest

The authors declare no conflict of interest.

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