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Rhizobia as complex biofertilizers for wheat: Biological nitrogen fixation and plant growth promotion

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Abstract:
The biological fixation of atmospheric nitrogen by rhizobia plays a key role in the cycle of ecosystems and their productivity. In agriculture, it is often used to increase the yield of legumes. We aimed to assess the stimulatory properties of three bacterial strains (*Ensifer meliloti* 441 B-219, *Ensifer mexicanus* B-4064, and *Rhizobium tropici* B-216) and their potential for promoting wheat growth under laboratory conditions.
The bacterial were obtained from the All-Russian Collection of Industrial Microorganisms (National Bioresource Center, Kurchatov Institute). To explore their potential for agronomic practices, we determined their stimulating properties and assessed antagonistic activity against such phytopathogens as *Fusarium graminearum* F-877, *Bipolaris sorokiniana* F-529, *Botrytis cinerea* F-1006, *Erwinia rhapontici* B-9292, and *Xanthomonas campestris* B-4102. Finally, we studied the effect of the strains on germination and the contents of photosynthetic pigments, nitrogen, and protein in the above-ground parts of wheat plants under laboratory conditions.
All the test rhizobia strains demonstrated various stimulating properties. In particular, they produced phytohormones, fixed nitrogen, solubilized phosphates and zinc, and synthesized ACC deaminase. The strains also exhibited pronounced antagonistic activity against *F. graminearum*, *B. sorokiniana*, and *Xanthomonas campestris*. According to the laboratory tests, the wheat seeds treated with *E. meliloti* 441 B-219 and *R. tropici* B-216 had longer shoots and roots, as well as higher contents of chlorophyll and carotenoids in some wheat varieties. *R. tropici* also had a strong positive effect on the weight of shoots and roots in all wheat varieties. *E. mexicanus* B-4064 exhibited a positive effect only on germination in some varieties. However, none of the strains had a significant effect on the nitrogen content.
The test rhizobia strains have significant potential for stimulating plant growth, but they do not contribute to a significant increase in nitrogen availability for wheat.

Keywords: *Triticum aestivum* L., nitrogen fixation, biofortification, phytohormones, siderophores, solubilization

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INTRODUCTION

A phytobiome is a community of microorganisms associated with a host plant. It plays a key role in maintaining the plant’s health and productivity in a changing environment. Microorganisms, such as bacteria and fungi, inhabit all plant organs but are particularly abundant in the rhizosphere [1, 2]. Bacteria are the most common and diverse microorganisms in this community [3] and they can be referred to as the second genome of the plant [4].

Root nodules of legumes are specialized organs that form as a result of interaction between the plant root system and bacteria of the *Rhizobium* genus. Root nodules have a unique microbiome dominated by rhizobia. These bacteria are crucial for the main stage of nitrogen metabolism, where atmospheric nitrogen (N_2) is reduced to ammonia (NH_3) in the presence of nitrogenase. This enzyme is a complex of MoFe dinitrogenase (molybdenum-iron protein), which is a catalyst, and dinitrogenase

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reductase (Fe protein). These two components are encoded by the genes nif, nif D, and nif K (for MoFe dinitrogenase), and the gene nif H (for Fe dinitrogenase reductase) [5–8].

A product of nitrogen fixation, ammonia is assimilated by the plant and converted into organic nitrogen compounds, which are necessary for growth and development. Thus, the specific relationship between the nodule root and its host plant is mainly controlled by the exchange of nitrogen (N) and carbon (C). The plant supplies the reduced carbon (in the form of carbohydrates) to the bacteria, which use it as energy to stimulate the nitrogen fixation process, and the nodules return the reduced nitrogen to the plant [9].

Biological nitrogen fixation provides legumes with an advantage under the conditions of nitrogen deficiency inthe soil, promoting their growth and development [10, 11]. Furthermore, biological nitrogen fixation by legumes is the main source of nitrogen in natural agroecosystems. The total nitrogen fixation is estimated at about 1.75×10^{11} kg worldwide, with nitrogen fixation by legumes accounting for 8.0×10^{10} kg and the industrial production of nitrogen fertilizers accounting for the remaining 8.8×10^{10} kg [12].

Nitrogen-fixing microorganisms are divided into three groups: free-living, associative, and symbiotic. Associative and symbiotic nitrogen fixers are found in the rhizosphere of both legumes and non-legumes [13, 14]. However, most studies have focused on the symbiosis of plants and nitrogen-fixing microorganisms resulting in the formation of root nodules. Research into root nodule metagenomes opens up new prospects for studying the composition and functional diversity of microbial species. However, we need to assess the effectiveness of isolated strains for the host plants, as well as their survivability in different soil conditions, before they can be used in sustainable agricultural practices. The symbiosis of rhizobia and legumes is a good model system for studying complex interactions. In particular, it can be used to study the mechanisms of interspecies recognition and the factors of nitrogen fixation. A better understanding of these interactions can improve crop management practices aimed at increasing the efficiency of atmospheric nitrogen in agriculture.

Diazotrophic bacteria play a key role in biological nitrogen fixation. They are also known to enhance plant growth and increase crop yield [15–18]. Studies show that numerous strains of *Rhizobia*, *Bradyrhizobia*, *Ensifer*, *Azotobacter*, *Azospirillum*, *Pseudomonas*, *Klebsiella*, and *Bacillus* have a beneficial effect on plant development and increase the yield of wheat, rice, and other crops. This is due to phytohormones (e.g., indoleacetic acid), siderophores, and organic acids, which stimulate the growth of stems and roots [17]. Gopalakrishnan *et al.* [19] found this effect on chickpea (*Cicer arietinum* L.). Some *Bradyrhizobial* strains isolated from the rhizosphere of rice and *Azorhizobium caulinodans* are able to fix nitrogen without entering into a symbiotic relationship with the plant [20], as well as under low oxygen conditions [21].

Baset *et al.* [22] reported the beneficial effects of rhizobia inoculation on rice, maize, and wheat. On the other hand, Gopalakrishnan *et al.* [23] and Das *et al.* [24] found that rhizobia can act as biological control agents against pathogenic fungi (*Rhizoctonia*, *Fusarium*, *Macrophomina*, and *Sclerotium*) by synthesizing hydrocyanic acid, antibiotics, and/or mycolytic enzymes. However, there has been inadequate research into the potential PGP properties of many other α-, β- and γ-proteobacteria associated with legume nodules and involved in nitrogen fixation processes. Studying these properties of diazotrophic bacteria, especially rhizobia, is important for selecting the most effective strains to increase crop yields.

In the last few years, significant efforts have been made to extend nitrogen fixation to cereals [25, 26]. Compared to symbiotic nitrogen-fixing bacteria, non-symbiotic bacterial diazotrophs are of limited agronomic use, although they account for about 30% of total biological nitrogen fixation [27]. They may be a source of fixed N in many terrestrial ecosystems [28]. This potential was supported by Pankiewicz *et al.* [29], who showed that *Setaria viridis* inoculated with an ammonia-producing strain showed robust growth under nitrogen-limiting conditions. Van Deynze *et al.* [30] reported that a Mexican maize cultivar fixed nitrogen at up to 82% when its aerial roots were colonized by symbiotic diazotrophic bacteria.

In this study, we aimed to assess the growth properties of three strains of rhizobia (*Ensifer meliloti* 441 B-219, *Ensifer mexicanus* B-4064, *Rhizobium tropici* B-216), as well as to study their potential for stimulating wheat growth under laboratory conditions. The effectiveness of these rhizobia strains as growth stimulants for wheat may promote their subsequent use in agrobiotechnology.

E. (Sinorhizobium) meliloti is an alphaproteobacterium, a gram-negative aerobic microorganism found in soil and capable of forming symbiotic interactions with nitrogen-fixing legumes of the genera *Medicago*, *Melilotus* and *Trigonella*. This bacterium is easily cultivated on various nutrient media and has great trophic capabilities in relation to sources of carbon, nitrogen, and phosphorus. Some *E. meliloti* strains do not colonize the plant's root system and therefore lack the ability to form symbiotic relationships [31]. Studies have shown that *E. meliloti* can act as an endophyte, colonizing various plant tissues, including leaves. There is a need to study the mechanisms of plant infection and intracellular reproduction of *E. meliloti*, including the regulatory mechanisms of the bacterial cell cycle and specific signaling molecules secreted by alfalfa, or lucern, plants (*Medicago* L.) [32, 33].

E. mexicanus is an aerobic, gram-negative, non-spore-forming rod. The ITTG R7 strain was isolated from the root nodules of narrow-leaved acacia (*Acacia angustissima* (Mill.) Kuntz) collected in Tuxtla Gutiérrez, Chiapas, Mexico [34]. Current scientific literature lacks a comprehensive description of the phenotypic and genotypic characteristics of *E. mexicanus*. Further

studies are needed to fully define its physiological, biochemical, and genetic properties, as well as analyze its nitrogen-fixing capacity and host plant specificity in comparison with other Ensifer species. The lack of detailed information on *E. mexicanus* limits our understanding of its role in ecosystems and its potential for agricultural use.

R. tropici is a poorly studied species of rhizobia isolated from root nodules of common bean and members of the *Leucaena* genus (woody legumes common in the tropics and subtropics). *R. tropici* is found in soil samples from various parts of the world. It can form symbiotic relationships with a wide range of legume host plants. The taxonomic group *R. tropici* includes a large number of strains with metabolic diversity [35]. They can synthesize a wide range of lipochito-oligosaccharides [36–38], which can have up to 60 structural configurations [39] depending on the bacterial species and environmental conditions. A lipo-chitooligosaccharide is a chain of 16 to 18 carbon atoms with a fatty acid residue that promotes the growth of non-legume plants. This is due to its ability to mimic the effects of plant hormones such as cytokinins and auxins [40], resulting in increased seed germination and resistance to pathogens [41].

STUDY OBJECTS AND METHODS

The bacterial strains under study were obtained from the All-Russian Collection of Industrial Microorganisms of the National Bioresource Center at the Kurchatov Institute National Research Center (Russia):

– *Ensifer meliloti* 441 B-219 isolated from the surface of alfalfa roots (Alma-Ata Region, 1961) and capable of producing nitragin;
– *Ensifer mexicanus* B-4064 capable of fixing atmospheric nitrogen; and
– *Rhizobium tropici* B-216 isolated from the surface of horse bean roots (Lithuanian Soviet Socialist Republic, 1967) and capable of producing nitragin.

To determine the cultural characteristics of the strains, a suspension of a bacterial strain with a low cell concentration was overlaid with a Drygalski spatula onto the medium containing 21.0 g/L of fermented meat hydrolysate (KhimMedService, Russia), 10.0 g/L peptone (ChemExpress, Russia), and 15.0 g/L agar-agar (LenReaktiv, Russia).

The morphological characteristics of the strains were determined by a microscopic examination of a fixed smear stained with methylene blue (LenReaktiv, Russia) at a total magnification of ×1000 [42].

To study the ability of the strains to produce indole-3-acetic acid, a cell suspension was prepared in 1% peptone water with a McFarland turbidity coefficient of 0.8–1.0 using a Densichek plus densitometer (BioMerieux, France). The cells were then inoculated on Luria-Bertani broth in the Miller modification (HiMedia Laboratories, India) in an amount of 3% of the nutrient medium. They were cultivated in an LSI-3016A shaker-incubator (Daihan Labtech, South Korea) for 24 h at 28 ± 2°C at 110 rpm. The cells were separated from the culture fluid by centrifugation for 10 min at 7500 rpm. Then, 1 mL of the Salkowski reagent (0.1 g iron (III) chloride (LenReaktiv, Russia) dissolved in 100 mL of 50% sulfuric acid (KhimKomponent, Russia)) was added to 1 mL of the resulting supernatant and kept for 30 min at 22 ± 2°C. At the same time, a control sample (nutrient medium with the Salkowski reagent) was prepared under the same conditions. The optical densities of the experimental and control samples were determined on a UV 1800 spectrophotometer (Shimadzu, Japan) at 535 nm [43]. The optical density of the sample (OD) was calculated according to Eq. (1):

$$OD = OD_{sp} - OD_c \quad (1)$$

where OD_{sp} is the optical density of the sample as read by the spectrophotometer; OD_c is the optical density of the control.

The amount of indole-3-acetic acid was determined using a graduated graph of standard solutions of indole-3-acetic acid (Dia-M, Russia) at concentrations from 0.5 to 5.0 μg/mL.

To study the ability of the strains to produce gibberellic acid, a cell-free culture fluid was prepared using the method described earlier. Then, 280 μL of zinc acetate (LenReaktiv, Russia) and potassium ferricyanide (LenReaktiv, Russia) was added to 2 mL of the culture fluid. The mixture was centrifuged for 15 min at 4500 rpm. Then, 1 mL of 30% hydrochloric acid (ChemExpress, Russia) was added to 1 mL of the resulting supernatant and kept for 75 min at 22 ± 2°C. The optical density was determined spectrophotometrically at 254 nm, with a 5% hydrochloric acid solution used as a control [44]. The amount of gibberellic acid was determined using a calibration graph of standard solutions of gibberellic acid (Dia-M, Russia) at concentrations from 0.5 to 5.0 mg/mL.

To study the ability of the strains to produce siderophores, a cell-free culture fluid was prepared using the method described earlier. Then, 1 mL of the resulting culture fluid was mixed with 1 mL of the CAS reagent (1.5 mL of a 0.016% solution of iron III chloride in a 10 M solution of hydrochloric acid mixed with 7.5 mL of a 1.21% solution of chromazurol S (ChemExpress, Russia) and made up to 100 mL with distilled water). The resulting solution was kept in a dark place for 30 min at 22 ± 2°C. At the same time, a control sample (nutrient medium with the CAS reagent) was prepared under the same conditions. The optical density was determined on a spectrophotometer at 630 nm. The concentration of siderophores (C_{sid}, %) was calculated using Eq. (2) [45]:

$$C_{sid} = \frac{OD_s - OD_c}{OD_c} \times 100 \quad (2)$$

where OD_s is the optical density of the sample; OD_c is the optical density of the control.

To study the ability of the strains to produce ammonia, a cell suspension was prepared using the method described earlier and inoculated on 1% peptone water. The cells were cultivated at 28 ± 2°C at 110 rpm for 48 h

and then separated from the culture fluid as described earlier. Then, 150 μL of Nessler's reagent (LenReaktiv, Russia) was added to 3 mL of the culture fluid and shaken. At the same time, a control sample (nutrient medium with Nessler's reagent) was prepared under the same conditions. The optical densities of the experimental and control samples were determined at 450 nm. The optical density of the sample was calculated according to Eq. (1). The amount of ammonia was determined by using a calibration graph of standard ammonium solutions at concentrations from 50 to 300 μg/mL [46].

To study the ability of the strains to fix nitrogen, a cell suspension was prepared in a physiological solution as described above and the resulting suspension was inoculated on Ashby broth (HiMedia Laboratories, India). The cells were cultivated and separated from the culture fluid under the conditions described for the ammonia production analysis. The amount of nitrogen in the culture fluid was measured with a Rapid N Cube nitrogen analyzer (Elementar, Germany) [44].

To study the ability of the strains to solubilize phosphates, a strain suspension was prepared as described above for the nitrogen fixation analysis. The resulting suspension was inoculated on Pikovskaya's broth (HiMedia Laboratories, India) and cultivated. The culture fluid was separated from the cells under the conditions described for the ammonia production analysis. Next, 1 mL of the culture liquid was mixed with 4.5 mL of a chloromolybdic acid solution (1.5 g ammonium molybdate (ChemExpress, Russia) dissolved in 40 mL of distilled water was mixed with 34.2 mL concentrated hydrochloric acid and made up to 100 mL with distilled water) and 25 μL of a chlorostannic acid solution (2.5 g tin chloride (ProfSnab, Russia) dissolved in 10 mL concentrated hydrochloric acid and made up to 100 mL with distilled water). The resulting solution was allowed to stand at 22–24°C for 15 min. The experimental and control optical densities were determined at 600 nm. The optical density of the sample was calculated by using Eq. (1). The amount of dissolved phosphorus was measured by using a calibration graph of a standard solution of dipotassium phosphate (LenReaktiv, Russia) at concentrations from 50 to 300 μg/mL [47].

To study the ability of the strains to solubilize zinc, a strain was inoculated in Petri dishes on the medium consisting of (g/L): 10.0 of glucose (ChemExpress, Russia), 1.0 ammonium sulfate (Dia-M, Russia), 0.2 potassium chloride (LenReaktiv, Russia), 0.1 potassium phosphate disubstituted, 0.2 magnesium sulfate (ChemExpress, Russia), 1.0 zinc oxide (LenReaktiv, Russia), and 15.0 agar-agar. The strain was cultured in a TSO-1/80 SPU thermostat (Smolensk Software Control Systems Design, Russia) for 4 days at 28 ± 2°C. A clearing zone around the colony indicated the strain's ability to solubilize zinc [48].

To study the ability of the strains to produce ACC deaminase, a strain was streaked in Petri dishes on the medium containing (g/L): 2.0 of ammonium sulfate, 4.0 potassium monobasic phosphate (LenReaktiv, Russia), 6.0 sodium dibasic phosphate (LenReaktiv, Russia), 0.2 magnesium sulfate, 0.001 ferrous sulfate (ChemExpress, Russia), 15.0 agar-agar, as well as solutions (1 mL each) of 0.01 g/L boric acid (KhimBaza, Russia), 0.01 g/L manganese sulfate (KhimBaza, Russia), 0.07 g/L zinc sulfate (ChemExpress, Russia), and 0.05 g/L copper sulfate (ChemExpress, Russia). The strain was cultured as described above. The growth of the culture indicated its ability to produce ACC deaminase [46].

To study the ability of the strains to produce biofilms, a strain was streaked in Petri dishes on the medium containing (g/L): 0.8 of Congo red (Dia-M, Russia), 50.0 sucrose (ChemExpress, Russia), 37.0 Brain Heart Infusion Broth (Micro-Lab, Russia), and 15.0 agar-agar. The strain was cultured as described above. The black color of the culture indicated its ability to produce biofilms [46].

The antagonistic activity of the bacterial strains was analyzed by using the following phytopathogens: *Fusarium graminearum* F-877, *Bipolaris sorokiniana* F-529, *Botrytis cinerea* F-1006, Erwinia rhapontici B-9292, and *Xanthomonas campestris* B-4102. The phytopathogenic fungi and bacteria were cultured in test tubes with potato-glucose agar (LenReaktiv, Russia) and GMF agar (LenReaktiv, Russia), respectively. A 24-h culture of a bacterium grown on a liquid Luria-Bertani medium (Miller's modification) was pour-plated into Petri dishes and incubated for 24 h at 28–30°C. Then, an agar block with the test culture was cut out and inserted into the well of an agar disk in the Petri dish with the phytopathogens. The phytopathogens were spread on agar washings. The bacterial suspension with a McFarland turbidity of 0.8 (1.5×10^8 CFU/cm^3) was law-plated. The Petri dishes were refrigerated for 8 h at 4°C to allow the metabolites of bacterial monocultures to diffuse from the block into the thickness of the agar with the test culture. Then, the phytopathogenic fungi were incubated in a thermostat at 26–28°C [49].

The laboratory tests were conducted according to State Standard 12038-84. For them, we used the varieties "Siberian Alliance", "The Hope of Kuzbass", and "In Memory of Aphrodite" of spring soft wheat (*Triticum aestivum* L.) and the microbiological fertilizer "Azofit" (SibBioPharm, Russia) based on nitrogen-fixing bacteria. The seeds were incubated in a Stellar-Fito LINE rack for plant growth (AWTech, Russia) at 20 ± 2°C.

On day 7 of incubation, we determined their germination (Eq. (3)). The length and weight of the sprout and root; as well as the contents of total chlorophyll, carotenoids, nitrogen, and protein.

$$G = \frac{N_{sd}}{N_{sp}} \times 100 \quad (3)$$

where G is the germination, %; N_{sd} is the number of seedlings; N_{sp} is the number of planted seeds.

To determine the pigment content, crushed leaves were extracted with 70% ethanol, kept for 30 min in a boiling water bath, and filtered. The sediment was filled with 70% ethanol and filtered. The pigment absorption was measured spectrophotometrically at 667 (total chlorophyll) and 480 nm (carotenoids) [50].

The nitrogen content in the above-ground part of the plants was determined on a Rapid N Cube nitrogen analyzer as described in the manual.

RESULTS AND DISCUSSION

The cultural and morphological characteristics of the test strains are presented in Fig. 1.

The bacterial strains *Ensifer meliloti* 441 B-219, *Ensifer mexicanus* B-4064, and *Rhizobium tropici* B-216 form white, round colonies. Their cells are rod-shaped and belong to gram-negative bacteria.

The growth-stimulating activity of the test microorganisms is presented in Figs. 2–6.

As can be seen in Fig. 2, *E. meliloti* 441 B-219 produced ACC deaminase and biofilms, *Ensifer mexicanus* B-4064 produced ACC deaminase and solubilized zinc, while *Rhizobium tropici* B-216 produced ACC deaminase.

As can be seen in Fig. 4, the amount of synthesized indolyl-3-acetic acid varied from 0.35 to 8.76 μg/mL, with *E. mexicanus* B-4064 showing the highest activity (8.76 μg/mL). The amount of gibberellic acid varied from 0.58 to 1.19 mg/mL, with *E. meliloti* 441 B-219 exhibiting the highest activity (1.19 mg/mL). The content of siderophores ranged from 43.2 to 51.5%, with *E. mexicanus* B-4064 displaying the highest activity (51.5%).

The ammonia content varied from 186 to 298 μg/mL, with *E. meliloti* 441 B-219 exhibiting the highest activity (298 μg/mL). The nitrogen content ranged from 175 to 194 μg/mL, with *R. tropici* B-216 showing the highest activity (194 μg/mL). The soluble phosphorus content varied from 100 to 229 μg/mL, with *E. mexicanus* B-4064 revealing the highest activity (229 μg/mL).

Our results were consistent with other studies. For example, Metuge *et al.* reported that *R. tropici* formed biofilms by synthesizing extracellular polymeric substances [51]. Imada *et al.* found that *R. tropici* CIAT 899 synthesized indole-3-acetic acid via the indole-3-pyruvate pathway. They also reported that the synthesis of this phytohormone was significantly inhibited by the presence of NH_4+ in the medium [52]. Sijilmassi *et al.* found that *R. tropici* CIAT 899 synthesized gibberellic acid and solubilized insoluble forms of phosphorus [53]. The ability of *R. tropici* to produce ACC deaminase might explain its ability to form rhizobial nodules. In addition, ACC deaminase plays a significant role in the antagonistic activity of the strains against phytopathogens [54].

Spini *et al.* reported that *E. meliloti* 3001 formed biofilms, as well as produced indole-3-acetic acid and siderophores [55]. Primo *et al.* found that the strains of *E. meliloti* had a varying ability to form agglomerates. For example, *E. meliloti* SR9 demonstrated high auto-aggregation phenotypes, while *E. meliloti* CU10 was not capable of forming biofilms [56]. The authors suggested that these differences may be associated with metabolic variations, especially with the production of exopolysaccharides. They observed a correlation between the phenotypic morphology of colonies (mucoid colonies are prone to biofilm formation) and their ability to synthesize exopolysaccharides. Our results confirm this relationship: *E. meliloti* 441 B-219, which exhibits a mucoid colony morphology, demonstrated the ability to form biofilms *in vitro*. In another study, Checcucci *et al.* described the ability of *Sinorhizobium meliloti* 1021 to synthesize ACC desamenase, which is probably involved in rhizosphere colonization or endophytic behavior [57]. According to Alami *et al.*, the strains of *E. meliloti* isolated from the plants growing in the abandoned tailings of the Zayda mine had the ability to solubilize insoluble forms of phosphorus [58]. This finding was confirmed by Borj *et al.* [59].

Current literature lacks data on the growth-promoting properties of *E. mexicanus*. Our study is the first to describe the ability of *E. mexicanus* B-4064 to produce phytohormones, siderophores, and ACC deaminase, as well as to mobilize zinc and phosphorus. These properties open up new possibilities for using this strain in agricultural technologies to increase crop yields. Our findings provide an insight into the biological potential of *E. mexicanus* and expand the range of potentially useful microorganisms for sustainable agriculture.

The antagonistic activity of the bacterial strains against the most common fungal and bacterial phytopathogens

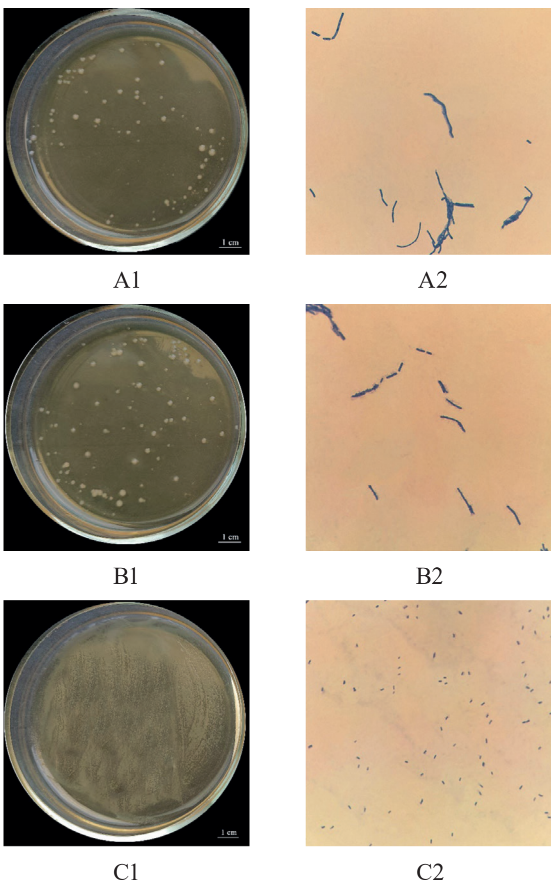


Figure 1 Cultural and morphological characteristics of *Ensifer meliloti* 441 B-219 (A), *Ensifer mexicanus* B-4064 (B), and *Rhizobium tropici* B-216 (C): 1 – cultural characteristics during growth on a solid medium after 24-h cultivation, 2 – morphological characteristics, with methylene blue staining (magnified ×1000)

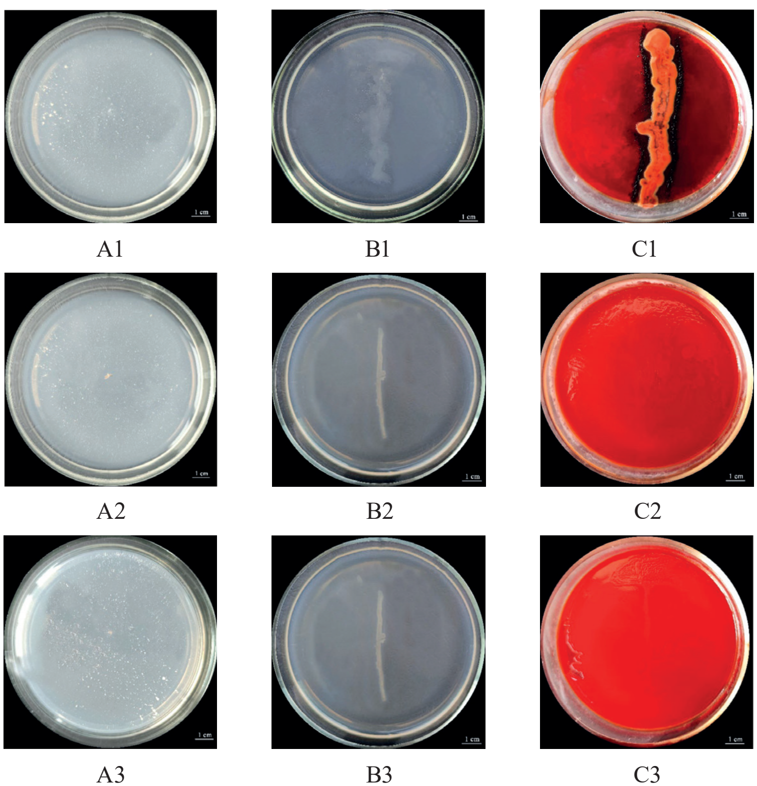
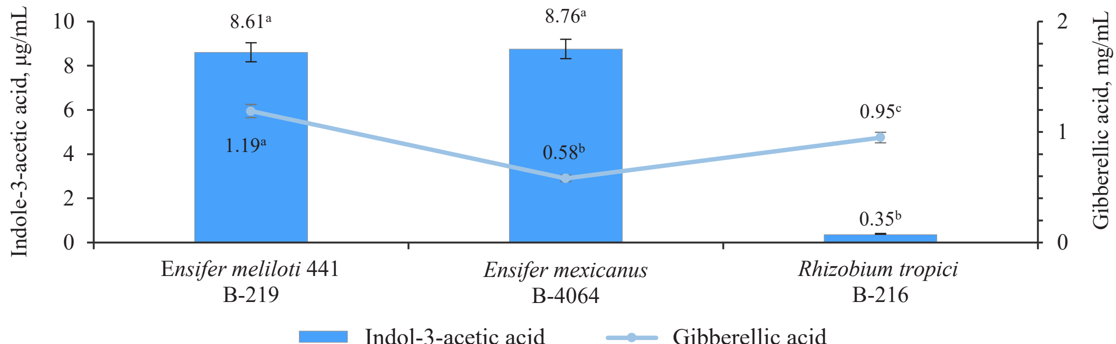
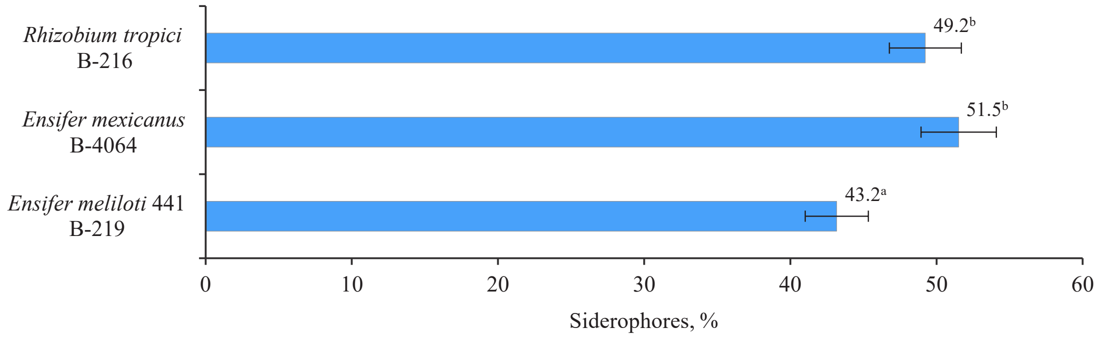


Figure 2 The ability of *Ensifer meliloti* 441 B-219 (1), *Ensifer mexicanus* B-4064 (2), and *Rhizobium tropici* B-216 (3) to solubilize zinc (A), produce ACC deaminase (B), and biofilms (C)



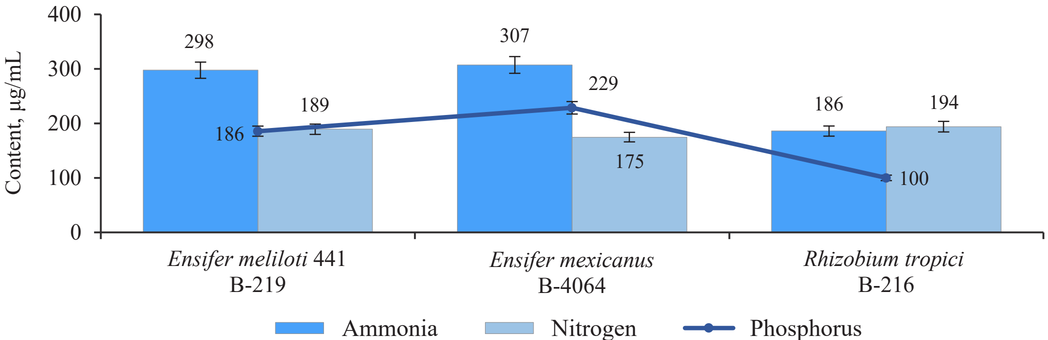
The letters to the right of the values indicate the significance of differences between the strains (within one parameter) according to ANOVA and the Scheffe test. The same letters mean no significance

Figure 3 Phytohormone-producing ability of the strains under study



The letters to the right of the values indicate the significance of differences between the strains (within one parameter) according to ANOVA and the Scheffe test. The same letters mean no significance

Figure 4 Siderophore-producing ability



The letters to the right of the values indicate the significance of differences between the strains (within one parameter) according to ANOVA and the Scheffe test. The same letters mean no significance

Figure 5 The ability of the strains to produce ammonia, nitrogen, and phosphorus

A1 B1 C1 1 (Control)

A2 B2 C2 2 (Control)

A3 B3 C3 3 (Control)

A4 B4 C4 4 (Control)

A5 B5 C5 5 (Control)

Figure 6 Antagonistic activity of *Ensifer meliloti* 441 B-219 (A), *Ensifer mexicanus* B-4064 (B), and *Rhizobium tropici* B-216 (C) against *Fusarium graminearum* F-877 (1), *Bipolaris sorokiniana* F-529 (2), *Botrytis cinerea* F-1006 (3), Erwinia rhapontici B-9292 (4), and *Xanthomonas campestris* B-4102 (5)

Table 1 Antagonistic activity of the bacterial strains

Strain	Antagonistic activity against, mm				
	Fusarium graminearum F-877	*Bipolaris sorokiniana* F-529	*Botrytis cinerea* F-1006	*Erwinia rhapontici* B-9292	*Xanthomonas campestris* B-4102
Ensifer meliloti 441 B-219	67 ± 3	65 ± 3	55 ± 2	23 ± 1	95 ± 5
Ensifer mexicanus B-4064	90 ± 5	95 ± 5	25 ± 1	–	77 ± 3
Rhizobium tropici B-216	95 ± 5	72 ± 4	40 ± 1	45 ± 2	95 ± 4

Table 2 Laboratory testing of strains on spring soft wheat varieties

Sample treatment	Germination, %	Average sprout length, mm	Average root length, mm	Average weight of one sprout, mg	Average weight of one root, mg
Siberian Alliance					
Water	87 ± 4	124 ± 5	94 ± 3	27.3 ± 1.0	20.9 ± 0.9
Azofit	87 ± 3	125 ± 6	97 ± 3	30.3 ± 1.3	23.4 ± 1.0
Ensifer meliloti 441 B-219	89 ± 4	158 ± 7	138 ± 5	34.5 ± 1.5	24.0 ± 1.0
Ensifer mexicanus B-4064	92 ± 4	133 ± 4	105 ± 4	32.4 ± 1.2	27.6 ± 1.2
Rhizobium tropici B-216	82 ± 4	163 ± 6	102 ± 4	39.0 ± 1.5	29.5 ± 1.0
The Hope of Kuzbass					
Water	88 ± 5	129 ± 5	91 ± 3	27.3 ± 1.2	24.4 ± 1.2
Azofit	89 ± 4	134 ± 6	89 ± 2	28.0 ± 1.3	22.2 ± 1.1
Ensifer meliloti 441 B-219	90 ± 5	172 ± 4	130 ± 4	28.6 ± 1.2	27.0 ± 1.0
Ensifer mexicanus B-4064	95 ± 3	156 ± 7	93 ± 4	32.0 ± 1.0	25.7 ± 1.3
Rhizobium tropici B-216	91 ± 4	191 ± 9	97 ± 5	38.9 ± 1.6	26.9 ± 1.2
In Memory of Aphrodite					
Water	76 ± 3	138 ± 5	91 ± 3	29.5 ± 1.2	22.8 ± 1.1
Azofit	75 ± 3	142 ± 6	91 ± 4	30.7 ± 1.5	21.7 ± 1.0
Ensifer meliloti 441 B-219	78 ± 2	157 ± 4	128 ± 4	31.3 ± 1.3	26.0 ± 1.2
Ensifer mexicanus B-4064	77 ± 4	145 ± 5	95 ± 2	33.1 ± 1.2	24.8 ± 1.0
Rhizobium tropici B-216	81 ± 4	199 ± 6	97 ± 4	41.2 ± 1.8	24.1 ± 1.3

(*Fusarium graminearum* F-877, *Bipolaris sorokiniana* F-529, *Botrytis cinerea* F-1006, *Erwinia rhapontici* B-9292, and *Xanthomonas campestris* B-4102) is shown in Fig. 6 and Table 1.

The bacterial strain *E. meliloti* 441 B-219 showed high antagonistic activity against *F. graminearum* F-877, *B. sorokiniana* F-529, and *X. campestris* B-4102. These results are consistent with those obtained by other scientists. For example, Batnini *et al.* reported that *S. meliloti* inhibited the growth of *Fusarium oxysporum*. In addition, they observed higher contents of proline and sucrose in the above-ground parts of the plants treated with the strain and infected with the phytopathogen. This indicates the ability of *S. meliloti* to mitigate biotic stress caused by *F. oxysporum* [60]. Our study revealed that *E. meliloti* strains have bactericidal properties.

E. mexicanus B-4064 exhibited high antagonistic activity against *F. graminearum* F-877, *B. sorokiniana* F-529, and *X. campestris* B-4102. However, it did not inhibit the growth of *E. rhapontici* B-9292.

The bacterial strain *R. tropici* B-216 showed high antagonistic activity against *F. graminearum* F-877, *B. sorokiniana* F-529, and *X. campestris* B-4102. According to Volpiano *et al.*, this may be due to the production of volatile organic compounds. The authors, who studied the strain *R. tropici* SEMIA 4077, reported that it inhibited 45% of *Sclerotium (Athelia) rolfsii* mycelium *in vitro* [61]. However, our study was the first to observe the inhibitory effect of the strain *R. tropici* B-216 on *F. graminearum*, *B. sorokiniana* and *X. campestris*. Thus, our results expand the understanding of its potential as a biological control agent.

Table 2 and Figs. 7–9 demonstrate the ability of the strains to stimulate the growth and development of the spring soft wheat varieties "Siberian Alliance", "The Hope of Kuzbass", and "In Memory of Aphrodite" under laboratory conditions.

The treatment of spring soft wheat with *E. meliloti* 441 B-219 did not have a significant positive effect on the germination or the weight of the sprout and root. However, it demonstrated a positive effect on the length of the sprout and root compared to the control group (treated with water and the microbial preparation Azofit). In the Siberian Alliance variety, the sprout length increased by 34 and 33 mm and the root length increased by 44 and 41 mm compared to the water-treated and Azofit-treated samples, respectively. In the Hope of Kuzbass variety, the sprout length, root length, and root weight increased by 40.5 mm, 40 mm, and 3.7 mg, respectively. In the variety In Memory of Aphrodite, the root length and root weight increased by 37 mm and 3.8 mg, respectively.

The strain *E. mexicanus* B-4064 had a significant positive effect on the germination of the Siberian Alliance and the Hope of Kuzbass varieties, increasing it

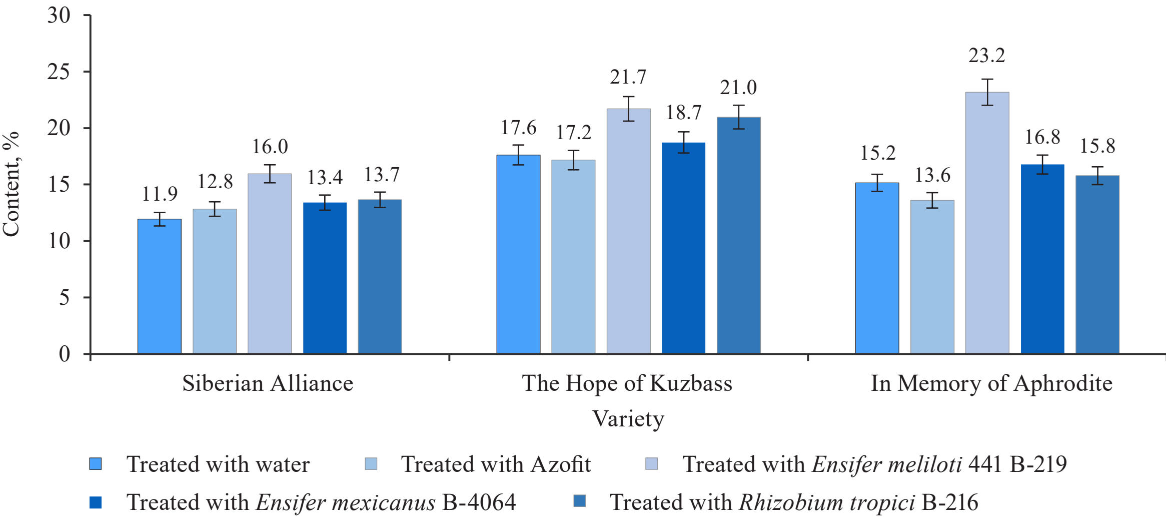


Figure 7 Total chlorophyll content in spring soft wheat sprouts

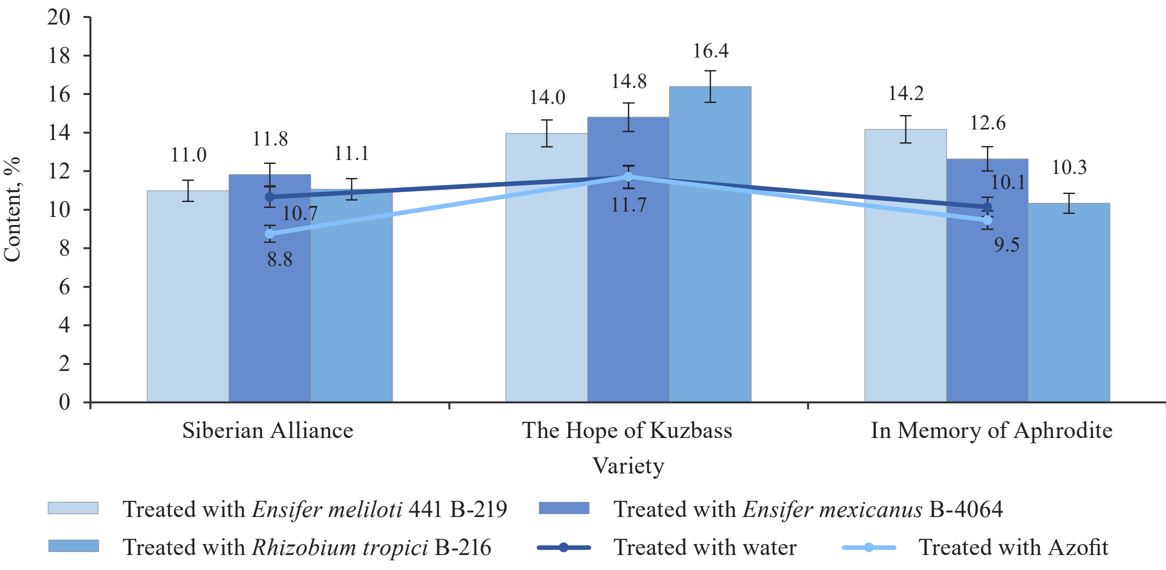


Figure 8 Carotenoid content in spring soft wheat sprouts

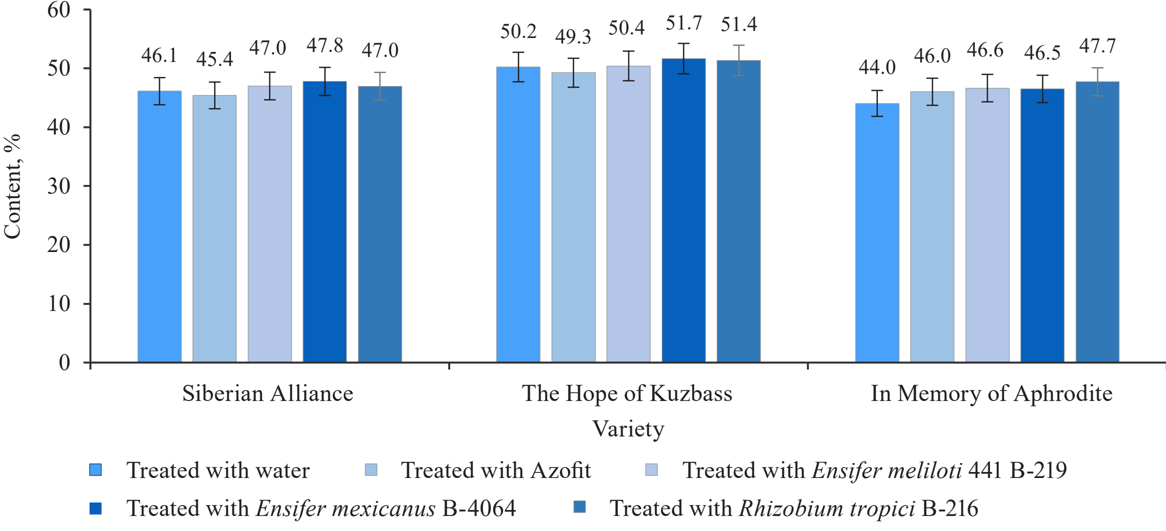


Figure 9 Nitrogen content in spring soft wheat sprouts

by 5 and 6.5%, respectively. However, no such effect was observed on the In Memory of Aphrodite variety.

The strain *R. tropici* B-216 had a positive effect on the three wheat varieties. In the Siberian Alliance variety, it increased the sprout length, sprout weight, and root weight by an average of 39.5 mm, 10.2 mg, and 7.4 mg, respectively, compared to the control group. In the Hope of Kuzbass variety, the strain increased the sprout length, sprout weight, and root weight by an average of 59.5 mm, 11.3 mg, and 3.4 mg, respectively. In the variety In Memory of Aphrodite, the strain increased the sprout length and weight by an average of 59 mm and 11.1 mg, respectively.

As can be seen in Fig. , *E. meliloti* 441 B-219 had a positive effect on the chlorophyll content in the wheat samples. In particular, the chlorophyll content amounted to 16.0, 21.7, and 23.2% of the mass in the Siberian Alliance, the Hope of Kuzbass, and In Memory of Aphrodite varieties, respectively. In the control group, the chlorophyll content reached 11.9, 17.6, and 15.2% in the water-treated samples of the three varieties, respectively; as well as 12.8, 17.2, and 13.6% in the Azofit-treated samples of the three varieties, respectively.

The *E. mexicanus* B-4064 strain did not have a significant positive effect on the total chlorophyll content in any of the spring soft wheat varieties. The *R. tropici* B-216 strain increased the chlorophyll content only in the Hope of Kuzbass variety (to 21.0%).

The *E. meliloti* 441 B-219 strain had a significant positive effect on the carotenoid content in the variety In Memory of Aphrodite (14.2%). In the control group, the carotenoid content reached 10.7, 11.7, and 10.1% in the water-treated samples of the Siberian Alliance, the Hope of Kuzbass, and In Memory of Aphrodite varieties, respectively; as well as 8.8, 11.7, and 9.5% in the Azofit-treated samples of the three varieties, respectively.

E. mexicanus B-4064 strain did not have a significant positive effect on the carotenoid content in any of the spring soft wheat varieties. *R. tropici* B-216 increased the carotenoid content only in the Hope of Kuzbass variety (to 16.4%).

The test rhizobia strains did not exert a significant positive effect on the nitrogen content in any of the three varieties of spring soft wheat. This might be due to the lack of nodules, specialized structures that ensure effective nitrogen fixation in legumes. The absence of nitrogen accumulation in the above-ground part of wheat may also be associated with its accumulation in the root system. However, even if rhizobia assumingly enhance nitrogen accumulation in the root system, their contribution to the total nitrogen content in wheat grain is quite insignificant. Nitrogen accumulation from vegetative organs during the pre-flowering period accounts for 50–95% [62] of the total nitrogen content in grain at harvest. Leaves and stems are the most important sources of nitrogen for grain [63], while the roots contribute only 10–15% [64]. Thus, nitrogen accumulation in the root system that could potentially result from rhizobia treatment is not a determining factor in agricultural practice.

Nevertheless, the rhizobia under study exerted a stimulating effect on the growth parameters and photosynthetic processes, contributing to longer shoots and roots, as well as higher chlorophyll and carotenoid contents. This might indicate that the rhizobia promote the formation of phytohormones, better absorption of other macro- and microelements, and changes in the morphogenesis of the root system. Further studies are needed to fully understand these mechanisms. In particular, there is a need to quantify phytohormones in plant tissues, study the microbial community of the rhizosphere, analyze the morphology of the root system, and determine the content of other nutrients. Controlled field experiments, which take into account the influence of abiotic factors, will allow us to assess the practical potential of the test rhizobia strains as biostimulants to increase wheat productivity. Our results can open up new directions for studying the interaction between rhizobia and cereal crops.

CONCLUSION

Symbiotic nitrogen fixation by rhizobia in association with legumes is a fundamental process of the nitrogen cycle that plays a key role in the productivity of agroecosystems. Although we fully understand the mechanisms of this interaction, the potential of rhizobia as growth promoters for non-legume plants needs to be studied further. In this study, we assessed the phytopromoting properties of three rhizobia strains: *Ensifer meliloti* 441 B-219, *Ensifer mexicanus* B-4064, and *Rhizobium tropici* B-216.

According to our results, *E. meliloti* 441 B-219 produced ACC deaminase and formed biofilms. The strain also produced phytohormones (8.61 μg/mL indolyl-3-acetic acid and 1.19 mg/mL gibberellic acid), siderophores (43.2%), ammonia (297.53 μg/mL), and nitrogen (189.22 μg/mL), as well as solubilized phosphates (185.71 μg/mL). In addition, *E. meliloti* exhibited pronounced antagonistic activity against *Fusarium graminearum* F-877, *Bipolaris sorokiniana* F-529, and *Xanthomonas campestris* B-4102.

E. mexicanus B-4064 synthesized ACC deaminase and solubilized zinc. The strain also produced phytohormones (8.76 μg/mL indolyl-3-acetic acid and 0.58 mg/mL gibberellic acid), siderophores (51.5%), ammonia (307.2 μg/mL), and nitrogen (174.78 μg/mL), as well as solubilized phosphates (228.57 μg/mL). *E. mexicanus* inhibited the growth of *F. graminearum* F-877, *B. sorokiniana* F-529, and *X. campestris* B-4102, but did not exhibit antagonistic activity against *Erwinia rhapontici* B-9292.

R. tropici B-216 synthesized ACC deaminase, phytohormones (0.35 μg/mL indolyl-3-acetic acid and 0.95 mg/mL gibberellic acid), and siderophores (49.2%). It also produced ammonia (185.87 μg/mL) and nitrogen (193.95 μg/mL), as well as solubilized phosphates (100.00 μg/mL). The strain had high antagonistic activity against the phytopathogens *F. graminearum* F-877, *B. sorokiniana* F-529, and *X. campestris* B-4102.

The spring wheat seeds treated with *E. meliloti* 441 B-219 and *R. tropici* B-216 had longer shoots and roots, as well as higher contents of chlorophyll and carotenoids (in some wheat varieties). *R. tropici* B-216 demonstrated the most pronounced positive effect on the length of shoots, as well as on the mass of the above-ground and underground parts in all wheat varieties. *E. mexicanus* B-4064 produced a significant positive effect only on seed germination. However, none of the bacterial strains had a statistically significant effect on the nitrogen content in the above-ground parts of the plants.

Although the rhizobia under study had no effect on the nitrogen content in the above-ground parts of the wheat plants, these strains have potential in agricultural practices due to their complex effect on the plants. In particular, they stimulate growth, increase stress resistance, and improve the absorption of nutrients such as phosphorus and zinc. Therefore, the use of the rhizobia can be more effective than the use of individual chemicals. In addition, the biological methods for increasing plant productivity are more environmentally friendly compared to the use of chemical fertilizers and pesticides.

However, further large-scale field trials under different agro-climatic conditions are needed to confirm the practical potential, long-term effects, and economic feasibility of using the rhizobia.

CONTRIBUTION

The authors were equally involved in writing the manuscript and are equally responsible for plagiarism.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding this publication.

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