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Research Article

Effect of Seed Inoculation with Actinomycetes and Rhizobium Isolated from Indigenous Soybean and Rhizosphere on Nitrogen Fixation, Growth, and Yield of Soybean

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The present study was initiated to determine whether isolates from soil and roots of soybean plants can express nitrogenase activity when grown in the absence of plant host. The study was conducted to answer the question "can benefit gained by" the interaction between Actinomycetes and Rhizobium symbiosis with legume. Thirty-five isolates identified as Rhizobium and twenty-one Actinomycetes were isolated from the rhizosphere of soybean plants and identified by morphological character, biochemical content identified. Fifty-six isolates were tested for their capabilities of N_2 fixation and siderophore production. The isolated rhizobacteria were grown in N-free media, and twelve of them showed a good growth on the Burk's N-free media. Almost all strains produced siderophores; however, the production level was very low, and only the strain AK 10 released considerable amounts of this metabolite. One strain of Actinomycetes was selected to test their interactions with Rhizobium. Coinoculation of Actinomycetes and Rhizobium produced synergic benefits on plant growth and get protection from the production of siderophore.

1. Introduction

Beneficial interaction effect between Rhizobium symbioses, especially in legumes, has been developed since the late 1800s. The use of microbial inoculants more involved Rhizobium and legume crops. Compounds produced by Rhizobium have been well researched and the number of Rhizobium strains that effectively form nodules has been identified. According to Garbaye and Frey-Klett [1], it is well established that some strains of Rhizobium can exhibit nitrogenase activity when grown in vitro on different media, but such activity has not been reported for Actinomycetes strains isolated from root nodules of N2-fixing nonlegumes [1].

Soybean growth and production can be promoted by improving soil fertility in terms of physical-chemical and biological characterization. In order to achieve such condition, endophytic microorganisms such as Rhizobium and Actinomycetes should be utilized to promote the growth in the production of soybean plants. In the last few years, Actinomycetes have been well known as commercial microorganisms that can produce antibiotics and other useful metabolites.

A previous study conducted reported that Actinomycetes of *Streptomyces lydicus* species WYEC108 colonizes the roots of peas *Pisum sativum*. Such root colonizations are potentially important for the improvement and growth of *Pisum sativum* plant by improving the process of the nodule formulation of Leguminosae plants because it promotes nodule formation. Actinomycetes *Streptomyces lydicus* WYEC108 infected root by affecting root nodulation of pea *Pisum sativum* by increasing the frequency of root nodulation; it is thought to occur at the rate of *Rhizobium* spp infection. *Streptomyces lydicus* also colonizes and then sporulates the surface of nodule cell [2]. The colonization may cause an increase in the average size of the nodule

formation and increase the strength of the nodule by increasing the assimilation of nodular iron and also improve soil nutrition [2].

Endophytic actinomycetes also play an important role in agriculture and forestry as they produce biopesticides instead of chemical pesticides. This helps to preserve the life and environment from toxic chemicals. Many people are interested in knowing about the existence of the Actinomycetes. There are two species of Actinomycetes which have been isolated and known to have a good bioactive activity, namely, *Frankia* sp and *Streptomyces scabies*. Genus *Frankia* is a nitrogen-fixing bacteria nonleguminous plant isolated in 1886 and *S. scabies* is found in 1890 as a phytopathogen [3]. As discussed by Sardi et al. [4], Actinomycetes endophyte isolated from the root surface sterilized from 28 plant species from northwest Italy and the most of the result of isolation process is *Streptoverticillium*, *Nocardia*, *Micromonospora*, and *Streptosporangium*.

Coomb et al. [5] isolated the endophytic Actinomycetes from the surface of the root tissue of healthy wheat plants (*Triticum aestivum* L.) collected from South Australia. They found that most isolates obtained are of the genus *Streptomyces* and of other species such as *Microbispora*, *Micromonospora*, and *Nocardiodes.* In addition, there are several studies that had isolated the endophytic Actinomycetes from other parts of the plant, such as De Araujo et al. [6] that isolated the endophyte from roots and leaves of corn. *Microbispora* sp is the most widely genus found, followed by *Streptomyces* sp. and *Streptosporangium* sp. Furthermore, Taechowisan et al. [7] has done the same process of isolation of the endophytic Actinomycetes from roots, stems, and leaves of herbaceous and woody plants in the sterile environment of Chiang Mai, Thailand, where the largest group of isolates they found were *Microbispora* sp, *Streptomyces*, *Nocardia*, and *Micromonospora*. Okazaki et al. [8] also found the genus *Microbispora* spp which isolated from leaves of the plant and the results were higher compared with isolates from the ground.

In 2010, several researchers tested thirty isolates for their capabilities of solubilizing/mineralizing sparingly phosphate sources, N2 fixation, and/or siderophore production, typical traits of the so-called plant growth-promoting rhizobacteria (PGPR). Phosphate-solubilizing ability was widely exhibited by the isolated. All of them produced acid phosphatase and thirteen of them produced alkaline phosphatase. Ten strains grew in N-free media. Almost all strains produced siderophores; however, the production level was in general very low and only the strain Thermobifida MCR24 released considerable amounts of this metabolite ([9], p. 210).

The objective of the present study was to isolate and identify Actinomycete strains with the intent of testing their abilities as a plant growth promoter. Therefore, the isolated Actinomycetes and Rhizobium were screened for their ability to produce siderophore typical secondary metabolites involved in rhizosphere colonization competence and tested for their capabilities to produce nitrogen in the absence of host plants. Then, the effects of selected Actinomycete strains on interaction with Rhizobium on plant growth and yield of soybean were also investigated.

2. Materials and Methods

Isolation process was carried out by taking root and soil samples of top soil at 0–20 cm in the three areas of soybeans rhizosphere in South Sulawesi, Indonesia. In each area, one healthy soybean was taken and placed in the plastic bag for isolation process. The soil samples around the rhizosphere area were collected in one bag and labeled based on the location criteria.

The method of serial dilution was employed to isolate the bacteria from rhizosphere of indegeneous soybeans. In this process, 10 grams of soil was dissolved in ninety mL of sterile water, and the solution was mixed for thirty minutes. One ml of suspension was inserted to the reaction tube containing nine mL of sterile water to get suspension with solution concentration at 10^{-2}. The same process was done for several levels of concentration, such as 10^{-3}, 10^{-4}, and then 10^{-6}, respectively.

Identification of bacterial isolates was conducted using methods developed by Schaaf et al. [10] as follows: characterization of morphology, determination of the morphological characteristics based on the shape and color of colonies on culture media nutrient agar (NA), and observations on a microscope. Identification of physiological and biochemical characteristics was performed through the following steps:

(a) Detect the criteria of gram reaction of tested bacteria by taking pure cultures using a needle loop and smeared on the glass objects. Then, the cultures on the object glass was added with aqueous KOH and stirred for 5 to 10 seconds. If the cultures showed slimy colonies, then they were identified as Gram-positive bacteria. On the contrary, the cultures which were not forming slimy colonies will be identified as Gram-negative bacteria.

(b) Morphology of different strains grown on tested media was analyzed for the mycelial organization and sporulation, under light microscopy. Cultural characteristics of Actinomycetes were studied on media natrium broth (NB). Colors of aerial and substrate mycelia were determined.

Identification of the molecular basis of the 10 isolates PGPR can be performed best by going through the following stages.

2.1. Testing Nitrogen Fixation. The ability of free nitrogen-binding bacteria carried by inoculation of Burk's N-free media should be tested. The isolates were able to grow, meaning that these isolates have the ability to bind free nitrogen for growth. Bacterial isolates were grown in media that Burk N-free for 24 hours at 28 ± 2°C. The pH value of the media is set to be 7.0 ± 0.1 before being autoclaved at 121°C for 15 minutes.

To measure the amount of N_2 fixation, bacterial isolates were tested by growing the bacteria in a liquid Burk's N-free medium and were placed on an orbital shaker for 24 hours at a temperature of 28 ± 20°C [8]. The method of Kjeldahl will be employed in order to measure nitrogen fixation [11].

Sample culture of bacteria was incorporated into the liquid Burk's N-free medium in 5 ml digestion tubes and then 1 g of a mixture of selenium and 3 ml of concentrated sulfuric acid were added and destructed up to 350°C temperature (3-4 hours). Destruction is considered complete when the steam out is white and when extracts obtained are clear (about 4 hours). The extracts were removed, cooled, and then diluted with distilled water up to exactly 50 ml and then shaken until it becomes homogeneous and left overnight so that the particles settle. The extract is used for the measurement of N by distillation.

Therefore, the extract was transferred into the boiling flask to release the NH3 by using Erlenmeyer flask containing 10 ml of 1% boric acid and added three drops of Conway (red) indicator. With a measuring cup, NaOH 40% as much as 10 mL into the boiling flask containing samples should be added and immediately cover with the lid. The samples were distillated until the container volume reached 50–75 ml (green). Distillation titration examples (Vc) and blank mo (Vb) then calculate the nitrogen content (%) by using the formula:

$$\text{Levels of nitrogen}\,(\%) = (\text{Vc} - \text{Vb}) \times \text{N} \times \text{bst}\,\text{N} \times 100\,\text{mg}. \quad (1)$$

Example 1

$$(\text{Vc} - \text{Vb}) \times \text{N} \times 14 \times 100 \cdot 500^{-1}$$
$$(\text{Vc} - \text{Vb}) \times \text{N} \times 2.8$$

Information

Vc, b = mL sample and blank titration
N = normality of standard solution H_2SO_4
14 = equivalent weight of nitrogen
100 = conversion to percentage (%)

Siderophore production was estimated by the method described by Reeves et al. [12]. Natrium Broth sterile medium was prepared as much as 100 ml in 250 ml Erlenmeyer flask. Bacterial isolates were inoculated with a loop into the medium and incubated at 37°C for 7 days. After 7 days of incubation, the liquid culture was centrifuged at 10,000 g for 20 minutes. The supernatant was used to estimate the siderophores. 20 ml of the culture supernatant was taken, and the pH was adjusted to 2.0 with dilute HCl. To 20 ml of the supernatant, 20 ml of ethyl acetate is added, and extraction is done twice. Prepare reagent of Hathway (1 ml of 0.1 M $FeCl_2$ and 1 mL of 0.1 N HCl is added to 100 ml of distilled water and added with 5 ml of reagent Hathway and absorbance was measured at 560 nm with sodium salicylate as standard in the range of 0.5 to 2 ppm to estimate salicylate kind of siderophore).

Twenty-one Actinomycete strains were preselected to investigate their plant growth promotion effects. Those are the higher ability of these strains for nitrogen testing and to produce siderophores. To investigate the plant promotion effects of these Actinomycetes, a pot experiment was carried out. This involved soybean plants (*Glycine max*. Linn) that were grown in a sterilized soil-sand mixture (300 ml pot). One of the Actinomycete strains was inoculated on the coated seed of soybean at a concentration of 10^4 cells/pot. There were thirteen treatments (one Actinomycete strain and one Rhizobium) with three replications for each treatment. Surface-sterilized seeds of soybean were germinated on the sterilized sand-soil substrate, and four seedlings were planted in each pot, watered as needed, and grown for 30 days. Pots were placed in a growth chamber in a randomized block design. After the growth period, the plants were harvested, dried at 60°C for 1 day, and weighed.

3. Results

3.1. Isolation and Identification of Actinomycetes and Rhizobium. The isolates are traditionally tentatively identified based on morphological criteria including the characteristics of the colonies on a petri dish, the morphology of the substrate and hypa air, spore morphology, and pigment produced [13], and also by referring to the Bergey's manual, [14], fifty isolates were identified (Figure 1) based on the morphological characteristics, which consist of 35 which have similarities in terms of colonies formed, colony color, colony edge, the surface of the colony, the colony growth rate, Gram test, the ability to form hypa air, spore morphology, and pigment produced. The other twenty-one isolates were similar in terms of the form of colonies, the edge of the colony, Gram test, and slime-forming bacteria (Figure 2).

The percentage (100%) of *Streptomyces* found in soil was very high compared with that of genera of Actinomycetes. The BLAST analysis was performed for all Actinomycetes. The results showed the confidence of sequencing analysis using the F1 and R5 primers correlating with those from the restriction endonuclease digestion technique used by Cook and Meyers [14].

3.2. Testing Nitrogen Fixation. As shown in Table 1, it is clear the growth ability for the twenty-one of identified Actinomycete strains and thirty-five of identified as Rhizobium on the Burk's N-free media. Ten strains were able to grow in these N-deficient media, suggesting that they could be nitrogen-fixing bacteria.

3.3. Siderophore Production. The production of siderophores was tested after evaluation of isolates on Burk's N-free media. The vast majority of isolates had produced two kinds of siderophores such as salicylate and catechol, as shown in Table 2, and it was clear that the solution showed the brown color on reaction tube containing tested isolates [15].

3.4. Interaction between Actinomycetes and Rhizobium. One of the isolated Actinomycetes was chosen in order to investigate their plant growth promotion effects on soybean plants. Consequently, the candidate of isolate was used to investigate some interactions with *Bradyrhizobium*. Interaction between Actinomycetes and Rhizobium experiments demonstrated the production of stems, seed, and mycelial development, as summarized in Table 3. The results of other

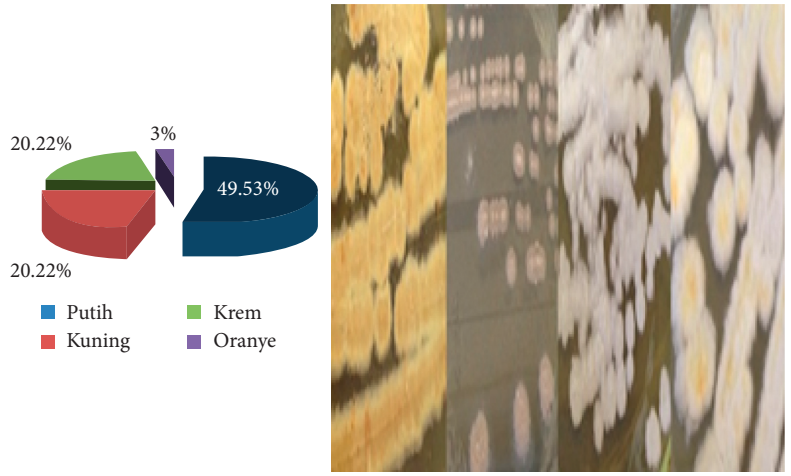

Figure 1: The percentage of bacteria identified as Gram-positive colony.

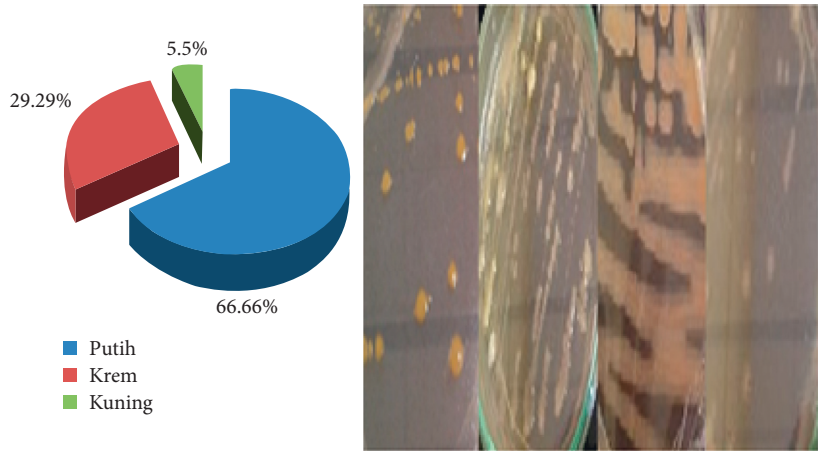

Figure 2: The percentage of bacteria identified as Gram-negative colony.

assays, which were designed to investigate any growth promotion effects using soybean plants, can be summarized as recorded in Tables 3 and 4. In general, the inoculated Actinomycetes coinoculate with Rhizobium and perform an excellent growth.

4. Discussion

Based on the results of electrophoresis by comparing the band of 16S ribosomal RNA gene amplification along with 234 base pairs which appeared on the test sample with 100 base pair marker on the left and right side, the PCR products were further sequenced to determine the basic sequence of each isolate tested and compared with the *Streptomyces* species found on GenBank (National Center for Biotechnology Information/NCBI) [16]. It is clear that the F1 and R5 primers, used for amplification of 16S rDNA gene of the Actinomycetes isolates in here reported experiments, confirmed their efficiency to amplify all sequences of 16S rDNA gene of target bacteria [14]. Results reported here maintain the use of the Cook methodology [14] because a partial 16S rDNA sequence of a new Actinomycetes isolate can be obtained quickly and at low cost to give a clear identification of the genus to which the isolate belongs, as can be seen in Table 5.

The invention of siderophores is a mechanism used by PGPR for rhizosphere colonization capability and has been revealed to be related with nitrogen fixation. Actually, symbiotic nitrogen fixers such as *Bradyrhizobium japonicum*, *Rhizobium leguminosarum*, and *Sinorhizobium meliloti* were reported as producers of siderophore [4, 16]. In the present study, the fifty-six isolates investigated showed positive result with regard to siderophore production, and ten isolates had an ability to fix nitrogen.

Several studies had proven that the differential effect can be shown by specific fungus whose the communities are related with the fungal hyphae, fungal spores, or/and with mycorrhizosphere of mycorrhizal plant [6, 18, 19, 20, 21]. It is clear that the condition of rhizosphere bacteria is under assortment to develop a fungus-specific character with the intention of giving a viable advantage during colonization of fungal surfaces. Bacteria-induced alterations to fungal development differentiation have been described in previous studies [23]. There were several effects of colonization already found by de Boer et al. [23], involving inhibition or promotion of germination; alterations to foraging

Table 1: Ability of Actinomycete isolates to grow in culture media without nitrogen source. The isolates may be diazotrophs based on their ability to grow in N-free media.

Isolate code	Nitrogen total (Kjeldahl) (%)	Growth on Burk's *N*-free media
AK 1	0.00	(−)
AK 2	0.00	(−)
AK 3	0.00	(−)
AK 4	0.00	(−)
AK 5	0.11	(+)
AK 6	0.00	(−)
AK 7	0.00	(−)
AK 8	0.00	(−)
AK 9	0.00	(−)
AK 10	0.00	(−)
AK 11	0.00	(−)
AK 12	0.00	(−)
AK 13	0.00	(−)
AK 14	0.00	(−)
AK 15	0.00	(−)
AK 16	0.20	(++)
AK 17	0.17	(+)
AK 18	0.00	(−)
AK 19	0.34	(+++)
AK 20	0.14	(+)
AK 21	0.17	(+)
RK1	0.00	(−)
RK2	0.00	(−)
RK3	0.00	(−)
RK4	0.00	(−)
RK5	0.00	(−)
RK6	0.00	(−)
RK7	0.00	(−)
RK8	0.00	(−)
RK9	0.00	(−)
RK10	0.00	(−)
RK11	0.00	(−)
RK12	0.20	(++)
RK13	0.00	(−)
RK14	0.00	(−)
RK15	0.00	(−)
RK16	0.00	(−)
RK17	0.00	(−)
RK18	0.00	(−)
RK19	0.00	(−)
RK20	0.00	(−)
RK21	0.17	(+)
RK22	0.14	(+)
RK23	0.00	(−)
RK24	0.00	(−)
RK25	0.20	(++)
RK26	0.00	(−)
RK27	0.34	(+++)
RK28	0.00	(−)
RK29	0.00	(−)
RK30	0.31	(+++)
RK31	0.00	(−)
RK32	0.00	(−)
RK33	0.00	(−)
RK34	0.22	(++)
RK35	0.00	(−)

(+++) = high nitrogen production. (++) = medium nitrogen production. (+) = low nitrogen production. (−) = no nitrogen production.

Table 2: Ability of Actinomycete isolates to produce both salicylate and catechol siderophore.

Isolate code	Siderophore production	
	Concentration of salicylate (mg/l)	Concentration of catechol (mg/l)
RK1	0.99	0.66
RK2	0.27	0.37
RK3	0.05	0.50
RK4	0.38	1.20
RK 5	0.58	0.52
RK 6	0.98	0.71
RK 7	0.58	0.60
RK 8	0.69	1.98
RK 9	0.62	1.21
RK 10	1.20	0.55
RK 11	0.64	0.41
RK 12	0.26	2.20
RK 13	0.18	0.62
RK 14	0.58	0.54
RK 15	0.17	1.63
RK 16	0.21	0.44
RK 17	0.25	0.95
RK 18	0.39	0.48
RK 19	0.41	0.56
RK 20	0.13	1.14
RK 21	0.49	0.12
RK 22	0.09	0.02
RK 23	0.41	0.26
RK 24	0.40	2.07
RK 25	0.43	0.95
RK 26	0.09	0.99
RK 27	2.82	0.92
RK 28	1.26	1.19
RK 29	0.30	1.03
RK 30	0.08	0.79
RK 31	0.03	0.76
RK 32	0.47	0.71
RK 33	0.06	0.24
RK 34	0.43	0.59
AK1	0.90	0.20
AK2	0.47	1.07
AK3	0.01	0.95
AK4	0.04	0.96
AK5	0.25	0.98
AK6	0.58	0.38
AK7	0.27	1.14
AK8	0.40	1.19
AK9	0.39	0.13
AK10	6.16	0.64
AK11	0.37	0.07
AK12	0.38	0.53
AK13	0.39	0.56
AK14	0.66	0.76
AK15	1.02	0.40
AK16	0.18	0.34
AK17	0.28	0.43
AK18	0.18	0.34
AK19	1.85	1.24
AK20	2.01	1.04
AK21	0.39	0.29
AK21	0.28	0.72
AK20	0.39	0.56
AK21	0.19	0.59

Table 3: Number of stems on treatment of Actinomycetes and Rhizobium.

Mean Actinomycetes	Mean Rhizobium			Total mean	NP BNJ α0.05
	$r1$	$r2$	$r3$		
Number of stems					
a0	3.83	4.33	4.67	4.28a	
a1	4.83	5.67	4.67	5.06a	0.92
a2	5.67	4.67	4.83	5.06a	
a3	5.83	5.67	6.00	5.83a	
Total mean	5.04x	5.08x	5.04x		
NP BNJ α0.05		0.80			

Mean values followed by the same letter are not significantly different. $P < 0.005$ (Tukey test).

Table 4: Number of soybean's pods on treatment.

Mean Actinomycetes	Mean Rhizobium			Total mean	NP BNJ α0.05
	$r1$	$r2$	$r3$		
Number of pods					
a0	30.50	38.00	34.50	34.33a	
a1	26.50	45.77	27.00	33.09a	8.31
a2	28.77	19.27	29.00	25.68a	
a3	39.50	38.00	43.27	40.26b	
Total mean	31.32x	35.26x	33.44x		
NP BNJ α0.05		7.19			

Mean values followed by the same letter are not significantly different. $P < 0.005$ (Tukey test).

Table 5: Number of seeds on the treatment of Actinomycetes and Rhizobium.

Mean Actinomycetes	Mean Rhizobium			Total mean	NP BNJ α0.05
	$r1$	$r2$	$r3$		
Number of seeds per plant					
a0	91.50	76.00	103.50	90.33[a]	
a1	53.00	91.50	61.00	68.50[a]	25.42
a2	63.17	49.75	67.67	60.19[a]	
a3	105.67	76.00	116.25	99.31a	
Total mean	78.33x	73.31x	87.10x		
NP BNJ α0.05		22.02			

Mean values followed by the same letter are not significantly different, $P < 0.005$ (Tukey test).

behavior, hyphal branching, growth, survival, reproduction, and exudates composition; and antibacterial metabolites production.

A novel plant-microbe interaction had been discovered by convincing the specific microbes which are known as *Streptomyces* sp and can induce a strong interaction between Glicyne max Merril and Rhizobium growing in soil. Numerous observations were made that show the importance of this interaction to the health of this legume when it was growing in soil. The microbial cells produced PHB in response to nitrogen limitation relative to carbon [15]. As a result of this, it induced the interaction between *Streptomyces* and Rhizobium when growing in the soil to perform a good growth of soybean and the yield of soybean. This condition supported by the significance of tricarboxylic acid cycle regulatory events like PHB synthesis and alanine synthesis also depends on the overall need to stabilize the oxidation-reduction state of pyridine nucleotides with the total carbon and nitrogen pools in the bacteroids [15].

The activities that promote plant growth could be an indirect way to influence Rhizobium and nodule symbiosis development. Actinomycete isolates improved both the number of stems and seeds. These Actinomycetes can be considered as PGPR because they may promote the colonization rate at different stages of bacterium-fungus-plant interactions. This ability was shown as the interaction between *Streptomyces* and *Pisum sativum* [23]. In the recent study in 2014, the interaction between Actinomycetes and Rhizobium on chickpea appears similar to the synergetic mutualistic interactions (Yadaf and Verma, 2014, p.70–77).

5. Conclusion

The reported experiments show that the target Actinomycete strains are able to improve plant growth and nutrition and benefit root colonization of soybean. Coinoculation with both types of microorganisms showed synergic effects at enhancing plant growth and nutrient acquisition. The results support the use of actinomycetes as plant growth-promoting and Rhizobium collaborator bacteria.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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