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Enhancing Symbiotic Nitrogen Fixation and Soybean Growth through Co-Inoculation with Bradyrhizobium and Pseudomonas Isolates

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Abstract: The present study was undertaken to explore acidotolerant rhizobial and non-rhizobial bacteria associated with root nodules of soybean (*Glycine max* L.). Genotypic and phenotypic characterization regarding nitrogen fixation, nodulation and other potentially plant growth-promotion traits were performed in several isolates. Influences of bacterial inoculation on symbiotic nitrogen fixation and soybean growth were evaluated through flask and pot experiments in a greenhouse. The highest nitrogen-fixing activity was recorded in isolate Bra6, which was closely related to *Bradyrhizobium diazoefficiens* based on 16S rDNA, *nifH*, and *nodD* gene sequences. All the non-rhizobial *Pseudomonas* isolates possessed multiple plant growth-promoting traits, with various hydrolytic patterns toward plant constituents. In sterile water agar-containing flasks, Bra6 + Pse2 treatment significantly ($p < 0.05$) increased the number of nodules, fresh weight, and dry weight of both root and shoot. This also led to the increment of most of the nutrients in the soybean plant compared with the uninoculated control or sole inoculation of Bra6. In non-sterile strongly acidic soil-containing pots, co-inoculation with Bra6 and various *Pseudomonas* isolates showed distinctively positive effects on symbiotic nitrogen fixation and soybean growth. The highest symbiotic nitrogen-fixing activity; root and shoot biomass; as well as N, P, K, Ca, Mg, S, Mn, Cu, and Zn contents of soybean plant were observed in Bra6 + Pse2 treatment. Synergistic symbiosis occurred through co-inoculation with *Bradyrhizobium* and *Pseudomonas* isolates, which further enhanced nutrients' acquisition and growth of soybean in the strongly acidic soils.

Keywords: acidotolerant rhizobia and non-rhizobia; co-inoculation; synergistic symbiosis; soybean

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1. Introduction

Studies on plant growth-promoting rhizobacteria (PGPR) have attracted much attention in the last decade. Application of PGPR with promising traits was considered as an environmentally friendly approach to sustain plant growth, which also reduced the requirement of chemical fertilization [1]. Among the beneficial bacteria, rhizobia that are able to form a symbiotic relationship with their host legumes are well studied [2,3]. To establish proper nodule formation, bacterial infection and nodule organogenesis need to be coordinated in plant tissue through signaling cascades [4]. It is a considerable challenge to understand the mechanisms by which legumes recognize and discriminate rhizobia from pathogens, as well as between distinct rhizobia species and strains that differ in their symbiotic performance [5]. Besides, a variety of PGPR have been demonstrated to enhance the legume–rhizobia symbiosis through improving the nodulation and nitrogen fixation [6].

Soybean (*Glycine max* L.) is an agronomically and economically relevant leguminous plant cultivated worldwide. It also showed potential to increase soil fertility when used as

green manure or cover crop [7–9]. Studies on bacterial community inside soybean nodules may help to explore effective participants involving in symbiotic nitrogen fixation and plant growth. It was reported that rhizobial subcommunities comprised mainly *Ensifer* and *Bradyrhizobium*, while the non-rhizobial groups were dominated by *Proteobacteria* and *Firmicutes* after screening nodule endophytes from fifty soybean fields [10]. Through the microbiome's analyses of nodules from nine soybean cultivars, Sharaf et al. [11] indicated that rhizobia were mainly composed of the family *Bradyrhizobiaceae*. Besides, the non-rhizobia such as *Pseudomonadaceae* and *Enterobacteriaceae* also dominated in soybean nodules. Within *Bradyrhizobiaceae*, three genera, namely, *Bradyrhizobium*, *Nitrobacter*, and *Tardiphaga*, were consistently detected in almost 193 root nodules of nine soybean plants [12]. The OTU analysis further demonstrated that *Bradyrhizobium* dominated during the entire stage of soybean growth, while the ratio of non-rhizobial bacteria showed an increasing trend as the soybean growth progressed [13]. From a meta-analysis of studies (from 1987 to 2018) conducted on soybean, Zeffa et al. [14] summarized that co-inoculation with *Bradyrhizobium* and PGPR resulted in a significant increase in nodule number (11.40%), nodule biomass (6.47%), root biomass (12.84%), and shoot biomass (6.53%). Among these PGPR, members belonging to genera *Azospirillum*, *Bacillus*, and *Pseudomonas* were more effective than *Serratia* in the improvement of nodule formation and soybean growth.

In our previous study, soybean intercropping was demonstrated to ameliorate tea-cultivated environments through changing bacterial communities as well as edaphic properties [9]. However, the roots of soybeans failed to form nodules during the experimental period, which was probably because of the strongly acidic nature of the tea plantation soils. As different species and strains differ in their symbiotic performance, it is crucial to explore effective rhizobia and non-rhizobia participating in the nodulation and growth promotion of soybean, especially in strongly acidic soils. In the present study, attempts were made to trap acidotolerant nodule-associated bacteria from soybean grown in a slightly acidic tea plantation. Effective rhizobial isolates were screened by nodulation test, while potentially plant growth-promoting and plant constituent-hydrolyzing traits were determined in non-rhizobial isolates. Effects of co-inoculation with beneficial rhizobial and non-rhizobial isolates on symbiotic nitrogen fixation and soybean growth were evaluated in sterile water agar (flask experiment) and non-sterile strongly acidic soils (pot experiment). This was used to test the hypothesis that acidotolerant nodule-inhabitants participated in the establishment of effective association with soybean, which further enhanced symbiotic nitrogen fixation and plant growth in the strongly acidic soils.

2. Materials and Methods

2.1. Isolation and Identification of Bacteria from Root Nodules

The green manure soybean (*Glycine max* L.) cultivar Tainan no. 7 was cultivated in a slightly acidic (pH 6.5) tea plantation for two months. Root nodules were collected and surface sterilized with 2% of sodium hypochlorite for 1 min followed by 70% of ethanol for 5 min. Another rinse with sterile water was repeated five times and the final rinse water was used to assess the sterilization process. Nodules were crushed and effluents were collected, serially diluted, and plated on acidic (pH 5) yeast extract mannitol agar (YEMA) [15]. Colonies that appeared within seven days of cultivation were picked and transferred several times to assure purity.

Genomic DNA was isolated from the three-day fresh cultures using the UltraClean Microbial Genomic DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's recommendations. 16S rDNA was amplified by PCR with bacterial universal primers, purified, and sequenced as described in [16], and bacterial identification was then performed by comparing the 16S rDNA sequences of isolates and their most closely related type strains using the EzBioCloud 16S-based ID function [17]. The 16S rDNA sequences obtained in this study were deposited in the NCBI GenBank database under Accession Numbers MZ798477–MZ798484.

2.2. Characterization of nifH and nodD Genes in Rhizobial Isolates

The *nifH* gene, which encodes the dinitrogenase reductase, was amplified by PCR with primer pair nifHF and nifHI [18]. The amplification cycles included an initial denaturation for 3 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 57 °C, and 2 min at 72 °C, with a final extension of 3 min at 72 °C. The *nodD* gene, which encodes the transcriptional regulator LysR family, was amplified by PCR with primer pair nodD7f and aboxp2 [19]. The amplification cycles included an initial denaturation for 30 s at 95 °C followed by 40 cycles of 30 s at 96 °C, 1 min at 53 °C, and 30 s at 72 °C, with a final extension of 5 min at 72 °C. Both the *nifH* and *nodD* amplicons were purified using DNA Clean & Concentrator Kit (ZYMO RESEARCH CORP., USA). Cycle sequencing and determination of the nucleotide sequence of the amplicons were performed at Genomics BioSci&Tech Ltd., Taiwan. The NCBI GenBank database was used to identify the closely related sequences with the amplicons using the BLASTn program. Concatenated sequences from 16S rDNA, *nifH*, and *nodD* genes were aligned using ClustalX program version 2 [20] and distances and clustering with the neighbor-joining method were performed using the software package MEGA version 11 [21]. The *nifH* and *nodD* gene sequences obtained in this study were deposited in the NCBI GenBank database under Accession Numbers MZ803186–MZ803191.

2.3. Nodulation Test of Rhizobial Isolates

Isolates that were classified as rhizobia were tested for their abilities to induce nodulation of soybean in a growth chamber (8 h day/16 h night cycles) at 25 °C for 45 days. Isolates were grown on YEM for three days, and cells were then harvested by centrifugation, washed twice, and resuspended in YEM to obtain bacterial suspension. Seeds were surface sterilized with 30% of hydrogen peroxide for 5 min, 70% of alcohol for 5 min, and rinsed five times with sterile water. After soaking in the bacterial suspension for 3 h, three seeds were placed on the filter paper. Filter papers were then rolled up and placed in the beaker containing 500 mL of sterile water. After one week of cultivation, seedlings were thinned down to one on each filter paper. Every two weeks, 10 mL of nutrient solution [15] was supplemented. After 25 days of cultivation, 5 mL of the bacterial suspension was re-inoculated in bacterial inoculation treatments. Regarding the uninoculated treatment, which represented the control, only 5 mL of YEM was supplemented. Six replications were conducted for each treatment. After 45 days of cultivation, the number of nodules and the growth of soybean including root length, shoot length, and number of leaves were determined. Symbiotic nitrogen-fixing activities of nodules were assessed as described in [22] using an acetylene reduction assay [23].

2.4. Determination of Plant Growth-Promoting and Plant Constituent-Hydrolyzing Traits of Isolate Bra6 and Non-Rhizobial Isolates

The three-day fresh colonies grown on YEMA were subjected to plant growth-promoting and plant constituent-hydrolyzing characterization. The free-living nitrogen-fixing activities of the isolates were assessed as described in [16] using an acetylene reduction assay [23]. To test for isolates with phosphate-solubilizing activities, colonies were picked and inoculated on/in tricalcium phosphate-containing medium, which was modified from Pikovskaya medium [24] and contained (L^{-1} distilled water: glucose 10 g, $(NH_4)_2SO_4$ 0.5 g, $Ca_3(PO_4)_2$ 5 g, KCl 0.2 g, $MgSO_4 \cdot 7H_2O$, 0.1 g, $MnSO_4 \cdot 5H_2O$ 0.001 g, $FeSO_4 \cdot 7H_2O$ 0.001 g, yeast extract 0.5 g, the pH was adjusted to 7.0 before autoclaving, and 2.5% of bacteriological agar powder was added when solid plates were prepared). To test for isolates with IAA-producing activities, colonies were picked and inoculated in YEM (pH 5 and 7) supplemented with 500 μg tryptophan mL^{-1}. The phosphate-solubilizing and IAA-producing activities of the five-day cultures were determined by the colorimetric method as described in [25]. Production of siderophore was evaluated on CAS agar plate as described in [26].

The decomposition of plant constituents was assessed by culturing the substrates tested into nutrient agar (Difco, Detroit, MI, USA) and inoculating the medium with isolates. The cellulolytic, pectinolytic, and amylolytic activities of isolates were evaluated by growing them on media supplemented with 0.5% of carboxymethylcellulose, pectin, and soluble starch, respectively. As for proteolytic activity determination, 1.5% of skimmed milk-containing nutrient agar was prepared. Bacterial cultures were cultivated at 30 °C for five days. The appearance of a clear zone was measured after the addition of specific reagents as described in [25].

2.5. Co-Inoculation Test in Sterile Water Agar through Flask Experiment

A preliminary co-cultural analysis was conducted to assess the compatibility of *Bradyrhizobium* isolate Bra6 and various *Pseudomonas* isolates. The three-day fresh cultures of each tested isolate were spread on the YEMA, and three paper discs filled with another tested cultures were placed on the same YEMA. After seven days of cultivation, the growths of both tested isolates were evaluated.

In the co-inoculation test, soybean was grown in sterile water agar-containing flasks for 60 days in greenhouse. The treatments included an uninoculated YEM control, sole inoculation of isolate Bra6, co-inoculation with Bra6, and various *Pseudomonas* isolates (Bra6 + Pse2, Bra6 + Pse3, Bra6 + Pse5, Bra6 + Pse6, Bra6 + Pse7). Seeds were surface sterilized with 2% of sodium hypochlorite for 5 min followed by 70% of ethanol for 5 min. Another rinse with sterile water was repeated five times and the final rinse water was used to assess the sterilization process. After soaking in the three-day cultures for 30 min, one seed was placed in the flask containing 200 mL of 0.7% water agar. After two weeks of cultivation, 2 mL of nutrient solution was supplemented. After three weeks of cultivation, 2 mL of the bacterial suspension was re-inoculated in bacterial inoculation treatments. As for the uninoculated treatment, only 2 mL of YEM was supplemented. Four replications were conducted for each treatment.

After 60 days of cultivation the number of nodules, fresh weight and dry weight (drying at 70 °C until a constant weight was obtained) of roots and shoots were determined. Plant N was measured by the Kjeldahl digestion method [27], as described in [28]. The P, K, Ca, Mg, S, Fe, Mn, Cu, Zn, B, Na, and Al contents of the dried and powdered plant materials were digested in a mixture (5:1) of nitric acid and perchloric acid at 120–180 °C [29] before measurements by ICP-OES ULTIMA 2C with a sequential JY 138 ULTRACE spectrometer (HORIBA Jobin Yvon Inc., Edison, NJ, USA).

2.6. Co-Inoculation Test in Non-Sterile Strongly Acidic Soils through Pot Experiment

In another co-inoculation test, soybean was grown in strongly acidic soil-containing pots for 65 days in greenhouse. The properties of the soils were analyzed according to the procedures described in [9], and the results were as follows: pH, 4.06; EC, 0.098 dS m^{-1}; total N, 0.11%; available P, 175.11 mg kg^{-1}; available K, 176.33 mg kg^{-1}; available Ca, 441.37 mg kg^{-1}; available Mg, 44.22 mg kg^{-1}; available Fe, 554.35 mg kg^{-1}; available Mn, 16.82 mg kg^{-1}; available Cu, 1.6 mg kg^{-1}; available Zn, 2.07 mg kg^{-1}; and available B, 0.5 mg kg^{-1}. The treatments included an uninoculated YEM control, sole inoculation of isolate Bra6, co-inoculation with Bra6, and various *Pseudomonas* isolates (Bra6 + Pse2, Bra6 + Pse3, Bra6 + Pse5, Bra6 + Pse6, Bra6 + Pse7).

Seeds were surface sterilized and soaked in the three-day cultures as described previously. Five seeds were sown at a depth of 2 cm in the plastic pot (diameter, 10 cm; depth, 8.5 cm) containing 500 g of acidic soils. The pots were sprinkled with water. After one week of cultivation, seedlings were thinned down to three in each pot. Every week, 10 mL of nutrient solution was supplemented. After two weeks of cultivation, 2 mL of the bacterial cultures was re-inoculated in bacterial inoculation treatments. Regarding the uninoculated treatment representing the control, only 2 mL of YEM was supplemented. Three replications were conducted for each treatment. After 65 days of cultivation, the number and fresh weight of nodules, as well as fresh weight and dry weight of roots and

shoots, were determined. Symbiotic nitrogen-fixing activity of nodules and nutrients of the above ground part were measured as described previously.

The results obtained for symbiotic nitrogen-fixing activity and plant growth were presented as mean values. One-way ANOVA (analysis of variance) and Duncan's test ($p < 0.05$) were used to evaluate the significant differences between treatments using XLSTAT statistical software (New York, NY, USA).

3. Results

3.1. Screening and Identification of Root Nodules-Associated Isolates

In this study, three rhizobial and five non-rhizobial isolates were obtained from acidic YEMA (Tables 1 and 2). 16S rDNA sequence analysis assigned them to the genus *Bradyrhizobium* and *Pseudomonas*, respectively. Isolate Bra4 shared 16S rDNA similarity of 100% with *Bradyrhizobium elkanii* USDA 76^T, *Bradyrhizobium pachyrhizi* PAC 48^T, *Bradyrhizobium tropiciagri* SEMIA 6148^T, *Bradyrhizobium brasilense* UFLA 03-321^T, and *Bradyrhizobium ripae* WR4^T. Isolate Bra6 was closely related to *Bradyrhizobium nanningense* CCBAU 53390^T (100%), *Bradyrhizobium centrosematis* A9^T (100%), *Bradyrhizobium guangxiense* CCBAU 53363^T (100%), and *Bradyrhizobium diazoefficiens* USDA 110^T (99.6%). Isolate Bra7 shared 16S rDNA similarity of 100% with *Bradyrhizobium diazoefficiens* USDA 110^T and *Bradyrhizobium niftali* CNPSo 3448^T. Isolate Pse2, Pse3, and Pse5 were most closely related to *Pseudomonas punonensis* CECT 8089^T (99.6%), *Pseudomonas mucoides* P154a^T (99.1%), and *Pseudomonas atacamensis* M7D1^T (99.9%), respectively. Isolate Pse6 and Pse7, which showed the same sequence of 16S rDNA, shared the highest similarity of 100% with *Pseudomonas glycinae* MS586^T.

Table 1. Blast result of 16S rDNA, *nifH*, and *nodD* gene sequence obtained from rhizobial isolates.

Isolate	Hit Taxon Name	Similarity		
		16S rDNA	*nifH*	*nodD*
Bra4	*Bradyrhizobium elkanii* USDA 76^T	1399/1399 (100%)	734/737 (99.6%)	328/329 (99.7%)
	Bradyrhizobium pachyrhizi PAC 48^T	1399/1399 (100%)	703/737 (95.4%)	311/329 (94.5%)
	Bradyrhizobium tropiciagri SEMIA 6148^T	1399/1399 (100%)	662/734 (90.2%)	284/327 (86.9%)
	Bradyrhizobium brasilense UFLA 03-321^T	1399/1399 (100%)	660/734 (89.9%)	NA
	Bradyrhizobium ripae WR4^T	1399/1399 (100%)	NA [†]	NA
Bra6	*Bradyrhizobium diazoefficiens* USDA 110^T	1394/1399 (99.6%)	737/737 (100%)	327/328 (99.7%)
	Bradyrhizobium nanningense CCBAU 53390^T	1399/1399 (100%)	687/737 (93.2%)	310/328 (94.5%)
	Bradyrhizobium centrosematis A9^T	1399/1399 (100%)	628/719 (87.3%)	NA
	Bradyrhizobium guangxiense CCBAU 53363^T	1262/1262 (100%)	626/737 (84.9%)	NA
Bra7	*Bradyrhizobium diazoefficiens* USDA 110^T	1384/1384 (100%)	737/737 (100%)	327/328 (99.7%)
	Bradyrhizobium niftali CNPSo 3448^T	1384/1384 (100%)	669/737 (90.8%)	303/328 (92.4%)

[†] NA: Sequence not available in NCBI GenBank database.

Table 2. Blast result of 16S rDNA sequence obtained from non-rhizobial isolates.

Isolate	Hit Taxon Name	Similarity
Pse2	*Pseudomonas punonensis* CECT 8089^T	1430/1436 (99.6%)
	Pseudomonas straminea JCM 2783^T	1428/1435 (99.5%)
	Pseudomonas argentinensis CH01^T	1428/1435 (99.5%)
Pse3	*Pseudomonas mucoides* P154a^T	1221/1232 (99.1%)
	Pseudomonas bijieensis L22-9^T	1420/1435 (99.0%)
	Pseudomonas gessardii DSM 17152^T	1422/1439 (98.8%)
	Pseudomonas mediterranea CFBP 5447^T	1422/1439 (98.8%)
Pse5	*Pseudomonas atacamensis* M7D1^T	1448/1450 (99.9%)
	Pseudomonas koreensis Ps 9-14^T	1447/1450 (99.8%)
Pse6	*Pseudomonas glycinae* MS586^T	1450/1450 (100%)
	Pseudomonas kribbensis 46-2^T	1448/1450 (99.9%)
Pse7	*Pseudomonas glycinae* MS586^T	1450/1450 (100%)
	Pseudomonas kribbensis 46-2^T	1448/1450 (99.9%)

3.2. Characterization of *nifH* and *nodD* Genes in Rhizobial Isolates

The *nifH* and *nodD* genes, which are associated with nitrogen fixation and nodulation, were successfully amplified and sequenced from three rhizobial isolates. Isolate Bra4 shared the highest sequence similarity of *nifH* (99.6%) and *nodD* (99.7%) with *Bradyrhizobium elkanii* USDA 76^{T} (Table 1). The sequence similarity of *nifH* and *nodD* between isolate Bra4 and other three closely related *Bradyrhizobium* species ranged from 89.9 to 95.4% and 86.9 to 94.5%, respectively. Both isolates Bra6 and Bra7 shared the highest sequence similarity of *nifH* (100%) and *nodD* (99.7%) with *Bradyrhizobium diazoefficiens* USDA 110^{T}. Concatenated sequences from 16S rDNA, *nifH*, and *nodD* genes supported the assignment of isolate Bra4 to *Bradyrhizobium elkanii*, while isolate Bra6 and Bra7 were affiliated with *Bradyrhizobium diazoefficiens* (Figure 1).

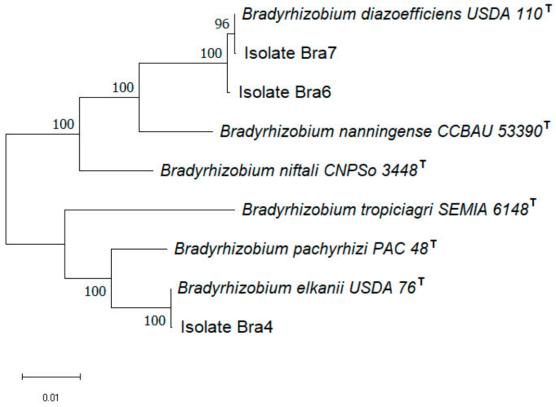


Figure 1. Neighbor-joining tree with bootstrapping 1000 replicates showing the phylogenetic relationship of three rhizobial isolates and their most closely-related species based on the concatenated sequences from 16S rDNA, *nifH*, and *nodD* genes.

3.3. Screening of Effective Rhizobial Isolates Inducing Root Nodulation

Three *Bradyrhizobium* isolates were separately served as inoculants to screen the effective partners forming symbiotic nitrogen fixation with soybean. After 45 days of cultivation, the formation of root nodules was found in all the treatments (Table 3). Compared with the uninoculated control, inoculation of isolate Bra4 or Bra7 showed a significantly ($p < 0.05$) higher number of nodules. There was no significant difference in root length, shoot length, and number of leaves between bacterial inoculation treatments. Isolate Bra6, which showed the highest symbiotic nitrogen-fixing activity, was selected and used in the following co-cultural analysis and co-inoculation experiments.

Table 3. Nodulation test of three rhizobial isolates.

Treatment	Root Length (cm)	Shoot Length (cm)	Number of Leaves	Number of Nodules	Nitrogen-Fixing Activity (nmol Ethylene h^{-1} $Nodule^{-1}$)
Control †	12.25 ± 3.66 a ‡	14.00 ± 3.34 a	17 ± 3 a	1 ± 1 c	0.10
Bra4	13.25 ± 1.60 a	12.77 ± 2.98 a	11 ± 1 b	5 ± 2 a	15.47
Bra6	12.25 ± 2.50 a	13.00 ± 4.10 a	13 ± 2 b	3 ± 2 bc	29.38
Bra7	13.83 ± 1.81 a	13.33 ± 1.03 a	11 ± 1 b	4 ± 1 ab	8.47

† Control: uninoculated treatment. ‡ Data represented as mean ($n = 6$) ± standard deviation. Different letters in the same column indicate significant differences ($p < 0.05$) in one-way ANOVA.

3.4. Plant Growth-Promoting and Plant Constituent-Hydrolyzing Traits of Isolate Bra6 and Non-Rhizobial Isolates

The in vitro tests were performed to evaluate plant growth-promoting potential of isolate Bra6 and five *Pseudomonas* isolates. All these isolates showed free-living nitrogen-fixing activities, which ranged from 0.027 to 0.337 nmol ethylene $tube^{-1}$ h^{-1} (Table 4). On tricalcium phosphate agar plates, the clear zones around colonies of all *Pseudomonas* isolates

were observed (Figure S1), and their phosphate-solubilizing activities ranged from 303.52 to 1391.43 μg mL^{-1}. The production of siderophore was also observed in all *Pseudomonas* isolates (Figure S2). In tryptophan-containing YEM, all of them showed IAA producing activities at both pH 5 and pH 7 conditions. The higher amounts of IAA production (between 32.91 and 80.24 μg mL^{-1}) were recorded at pH 7 in all these isolates.

Table 4. Plant growth-promoting traits of the tested isolates.

Isolate	Nitrogen-Fixing Activity (nmol Ethylene $Tube^{-1}$ h^{-1})	Phosphate-Solubilizing Activity (μg mL^{-1})	Production of Siderophore (Halo Diameter/Colony Diameter)	Production of IAA in Tryptophan-Containing YEM (μg mL^{-1})	
				pH 5	pH 7
Bra6	0.337	ND †	ND	ND	ND
Pse2	0.091	311.33	1.11	3.30	32.91
Pse3	0.040	303.52	1.26	3.22	35.11
Pse5	0.081	1391.43	1.24	8.70	73.76
Pse6	0.053	1212.86	1.26	8.61	78.29
Pse7	0.027	1230.71	1.29	11.22	80.24

† ND: not detectable.

Considering the plant constituent-hydrolyzing traits, all these isolates failed to produce a clear zone on carboxymethylcellulose-containing medium (Figure S3). Three isolates, namely, Pse5, Pse6, and Pse7, were positive in pectin degradation (Figure S4). Only isolate Bra6 and Pse2 showed amylolytic capabilities on soluble starch-containing medium (Figure S5). All these isolates except for Bra6 and Pse2 showed proteolytic capability, as revealed by the apparent clear zone around colonies on skimmed milk-containing medium (Figure S6).

3.5. Influence of Co-Inoculation on Nodulation and Soybean Growth in Sterile Water Agar Evaluated through Flask Experiment

The compatibility of *Bradyrhizobium* isolate Bra6 and various *Pseudomonas* isolates was evaluated through co-cultural analysis on the YEMA. Within seven days of cultivation, there was no apparent growth inhibition between isolate Bra6 and the tested *Pseudomonas* isolates (Figure 2). Therefore, all of them were further used in the following co-inoculation experiment.

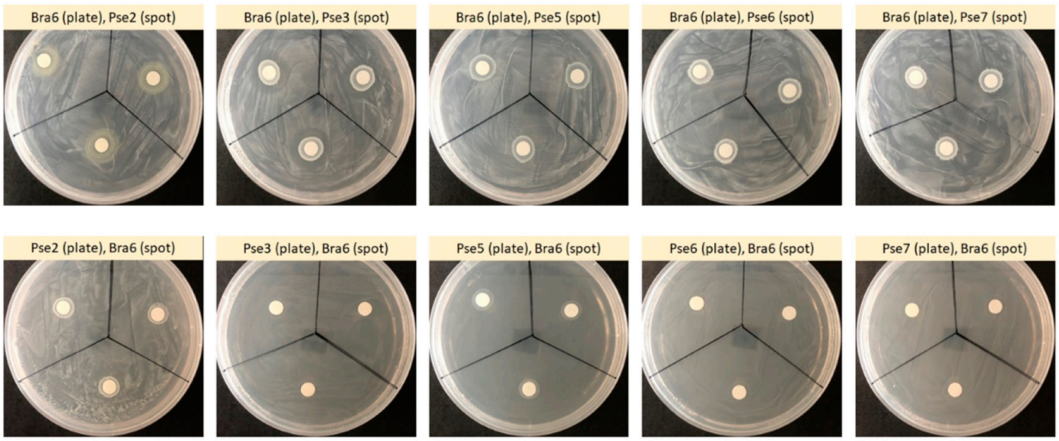


Figure 2. Co-cultural analysis of *Bradyrhizobium* isolate Bra6 and various *Pseudomonas* isolates. Each tested isolate was spread on the YEMA (labeled as plate in the parentheses), and three paper discs filled with another tested cultures (labeled as spot in the parentheses) were placed on the same YEMA.

In sterile water agar-containing flasks, there was no nodule formation during two months of soybean cultivation in the uninoculated control (Figure 3a). In contrast, sole inoculation of Bra6 or co-inoculation with Bra6 and various *Pseudomonas* isolates showed different influences on nodule development. The higher number of nodules was observed in both Bra6 + Pse2 and Bra6 + Pse6 treatments, while it was the lowest in Bra6 + Pse7

treatment. Compared with the uninoculated control, the fresh weight of root and shoot significantly ($p < 0.05$) increased by 22% and 32%, respectively, in Bra6 + Pse2 treatment (Figure 3b). This treatment also showed increments of 34% and 29% in the dry weight of root and shoot, respectively (Figure 3c). Besides, there were 25% and 24% increases in the dry weight of shoot in Bra6 + Pse3 and Bra6 + Pse6 treatments, respectively, compared with the uninoculated control.

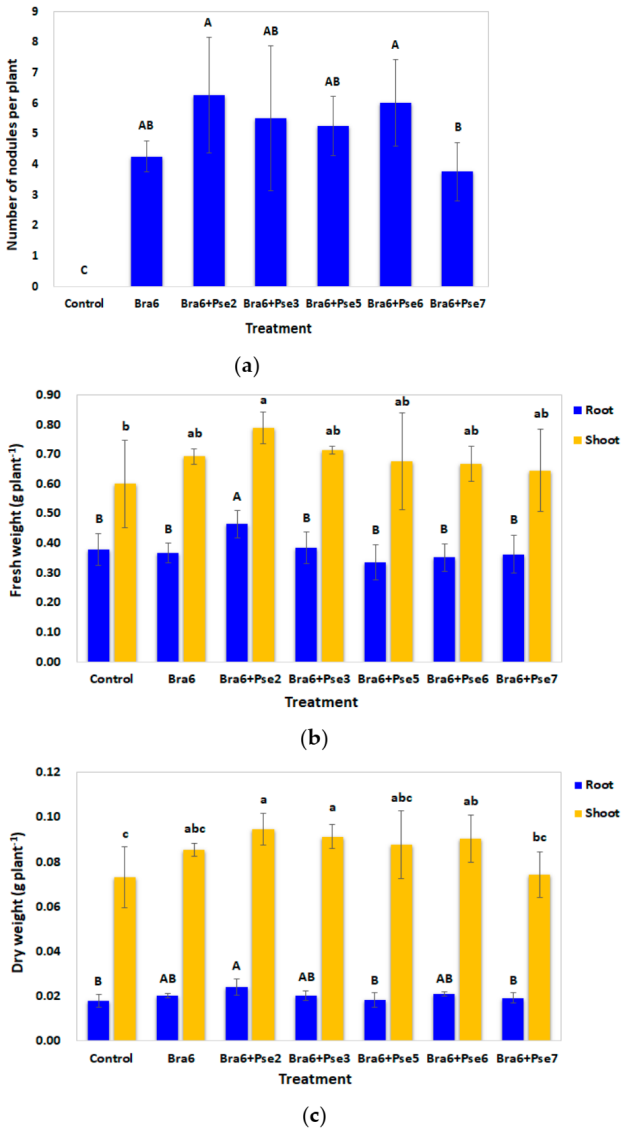


Figure 3. Effects of bacterial inoculation on the (**a**) number of nodules, (**b**) fresh weight of root and shoot, and (**c**) dry weight of root and shoot in sterile water agar evaluated through flask experiment. Control, uninoculated treatment. Error bar, mean ($n = 4$) ± standard deviation. Different letters above bars indicate significant differences ($p < 0.05$) in one-way ANOVA.

Bra6 + Pse2 and Bra6 + Pse3 treatments significantly ($p < 0.05$) increased P, K, Ca, Mg, S, Mn, Zn, and B contents of soybean plant compared with the uninoculated control (Table 5). The highest Fe and Al contents were also recorded in Bra6 + Pse2 treatment. Bra6 + Pse5 treatment significantly increased the K, Ca, Mg, Mn, Zn, and B contents. Besides, higher K, Ca, Mg, S, and B contents were also recorded in Bra6 + Pse6 treatment compared with the uninoculated control. Among all these treatments, inoculation with Bra6 and Pse7 led to the lowest nutrient contents of soybean plant, no matter which element was used for comparison.

Table 5. Effects of bacterial inoculation on the nutrient contents of soybean plant grown in sterile water agar-containing flasks.

Treatment	N	P	K	Ca	Mg	S	Fe	Mn	Cu	Zn	B	Na	Al
							mg plant^{-1}						
Control †	4.8 ± 0.7 a ‡	0.9 ± 0.1 b	1.9 ± 0.3 c	0.5 ± 0.1 c	0.5 ± 0.1 b	0.5 ± 0.1 c	0.027 ± 0.004 bc	0.003 ± 0.000 c	0.002 ± 0.000 bc	0.006 ± 0.001 b	0.008 ± 0.001 c	0.4 ± 0.1 a	0.014 ± 0.002 b
Bra6	4.8 ± 0.1 a	0.9 ± 0.0 b	2.3 ± 0.0 b	0.6 ± 0.0 bc	0.6 ± 0.0 b	0.6 ± 0.0 b	0.029 ± 0.001 ab	0.004 ± 0.000 bc	0.002 ± 0.000 c	0.006 ± 0.000 b	0.008 ± 0.000 bc	0.3 ± 0.0 b	0.016 ± 0.000 a
Bra6 + Pse2	4.5 ± 0.1 ab	1.2 ± 0.0 a	2.4 ± 0.1 ab	0.7 ± 0.0 a	0.7 ± 0.0 a	0.7 ± 0.0 a	0.031 ± 0.001 a	0.004 ± 0.000 ab	0.002 ± 0.000 ab	0.007 ± 0.000 a	0.011 ± 0.000 a	0.4 ± 0.0 a	0.017 ± 0.001 a
Bra6 + Pse3	4.8 ± 0.2 a	1.2 ± 0.1 a	2.7 ± 0.1 a	0.7 ± 0.0 a	0.7 ± 0.0 a	0.6 ± 0.0 ab	0.024 ± 0.001 c	0.004 ± 0.000 ab	0.002 ± 0.000 a	0.007 ± 0.000 a	0.011 ± 0.001 a	0.4 ± 0.0 a	0.013 ± 0.001 b
Bra6 + Pse5	4.8 ± 0.7 a	1.0 ± 0.2 b	2.5 ± 0.4 ab	0.7 ± 0.1 a	0.7 ± 0.1 a	0.6 ± 0.1 bc	0.019 ± 0.003 d	0.004 ± 0.001 a	0.001 ± 0.000 c	0.008 ± 0.001 a	0.009 ± 0.001 b	0.3 ± 0.0 b	0.011 ± 0.002 c
Bra6 + Pse6	4.0 ± 0.4 bc	1.0 ± 0.1 ab	2.4 ± 0.2 ab	0.7 ± 0.1 ab	0.7 ± 0.1 a	0.6 ± 0.1 b	0.017 ± 0.002 d	0.003 ± 0.000 c	0.002 ± 0.000 c	0.006 ± 0.001 b	0.009 ± 0.001 b	0.4 ± 0.0 a	0.010 ± 0.001 c
Bra6 + Pse7	3.6 ± 0.4 c	0.6 ± 0.6 c	1.4 ± 0.1 d	0.4 ± 0.0 d	0.4 ± 0.0 c	0.3 ± 0.0 d	0.012 ± 0.001 e	0.002 ± 0.000 d	0.001 ± 0.000 d	0.003 ± 0.000 c	0.005 ± 0.001 d	0.2 ± 0.0 c	0.008 ± 0.001 d

† Control: uninoculated treatment. ‡ Data represented as mean (n = 4) ± standard deviation. Different letters in the same column indicate significant differences ($p < 0.05$) in one-way ANOVA.

3.6. Influence of Co-Inoculation on Nodulation, Symbiotic Nitrogen Fixation, and Soybean Growth in Non-Sterile Strongly Acidic Soils Evaluated through Pot Experiment

In non-sterile strongly acidic soil-containing pots, the number and fresh weight of nodules were significantly ($p < 0.05$) higher in bacterial inoculation treatments than in the uninoculated control after two months of cultivation (Figure 4a,b). There was no significant difference in nodule formation between sole inoculation of Bra6 or co-inoculation with Bra6 and various *Pseudomonas* isolates. Compared with the uninoculated control, co-inoculation with Bra6 and various *Pseudomonas* strains also increased symbiotic nitrogen-fixing activity to a significant level (Figure 4c). The highest nitrogen-fixing activity was recorded in Bra6 + Pse2 treatment. In consideration of soybean growth, sole inoculation of Bra6 increased the fresh weight of root and shoot by 13% and 8%, respectively (Figure 4d). Significant increments of 33%–53% in root fresh weight were observed in all the co-inoculation treatments, and there were 12% and 12% increases in shoot fresh weight in Bra6 + Pse2 and Bra6 + Pse3 treatments, respectively, compared with the uninoculated control. The highest dry weight of root and shoot was recorded in Bra6 + Pse2 treatment, with 26% and 13% increments compared with the uninoculated control (Figure 4e).

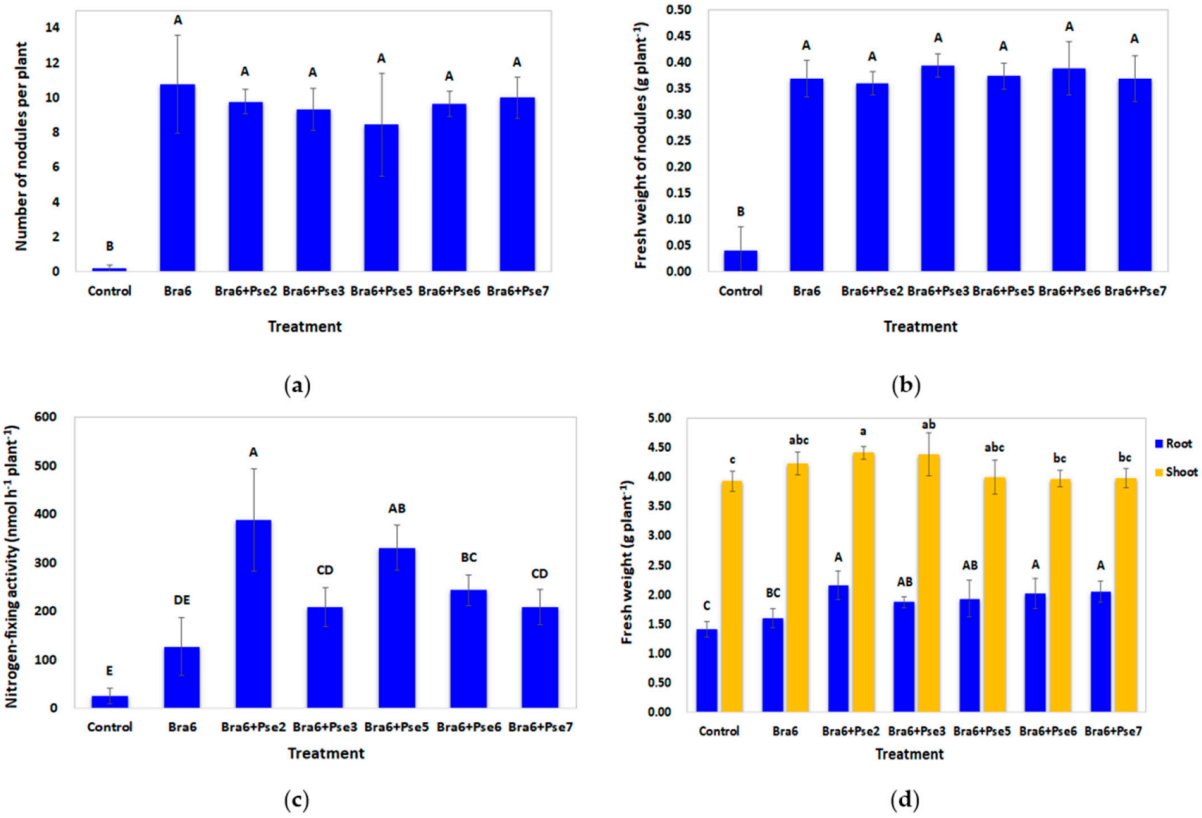
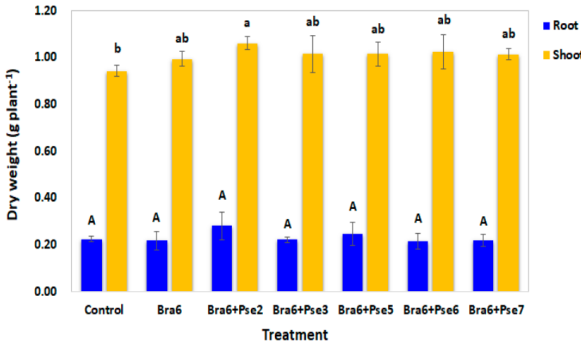


Figure 4. *Cont.*



(**e**)

Figure 4. Effects of bacterial inoculation on the (**a**) number of nodules, (**b**) fresh weight of nodules, (**c**) symbiotic nitrogen-fixing activity, (**d**) fresh weight of root and shoot, and (**e**) dry weight of root and shoot in strongly acidic soils evaluated through pot experiment. Control, uninoculated treatment. Error bar, mean (n = 3) ± standard deviation. Different letters above bars indicate significant differences ($p < 0.05$) in one-way ANOVA.

Compared with the uninoculated control, sole inoculation of Bra6 significantly ($p < 0.05$) increased the N, K, Ca, Mn, Cu, and B contents of soybean plant (Table 6). Co-inoculation with Bra6 and Pse2 showed a profound increment in most of the nutrients, with significantly higher N, K, Ca, Mg, S, Mn, Zn, and Na contents compared with Bra6-inoculated treatment. Other co-inoculation treatments also contributed to the accumulation of N, K, Ca, and Mn to a significant level compared with the uninoculated control.

Table 6. Effects of bacterial inoculation on the nutrient contents of soybean plant grown in strongly acidic soils-containing pots.

Treatment	N	P	K	Ca	Mg	S	Fe	Mn	Cu	Zn	B	Na	Al
							mg plant^{-1}						
Control †	8.3 ± 1.3 c ‡	2.0 ± 0.1 ab	9.6 ± 0.3 e	6.0 ± 0.2 c	2.7 ± 0.1 c	2.7 ± 0.1 ab	0.161 ± 0.004 a	0.426 ± 0.011 d	0.011 ± 0.000 bc	0.055 ± 0.001 bc	0.032 ± 0.001 de	0.2 ± 0.0 d	0.194 ± 0.005 a
Bra6	19.0 ± 0.3 b	2.1 ± 0.1 a	12.4 ± 0.4 bc	7.3 ± 0.2 b	3.0 ± 0.1 bc	2.6 ± 0.1 b	0.159 ± 0.005 a	0.559 ± 0.017 b	0.012 ± 0.000 a	0.059 ± 0.002 b	0.041 ± 0.001 a	0.2 ± 0.0 cd	0.158 ± 0.005 bc
Bra6 + Pse2	24.1 ± 0.8 a	2.1 ± 0.1 a	13.9 ± 0.3 a	8.1 ± 0.2 a	3.3 ± 0.1 a	2.8 ± 0.7 a	0.137 ± 0.003 b	0.605 ± 0.015 a	0.012 ± 0.000 a	0.067 ± 0.002 a	0.036 ± 0.001 bc	0.2 ± 0.0 b	0.169 ± 0.004 b
Bra6 + Pse3	18.9 ± 3.8 b	2.0 ± 0.2 ab	12.8 ± 1.0 b	7.1 ± 0.6 b	3.1 ± 0.2 ab	2.6 ± 0.2 ab	0.099 ± 0.008 e	0.498 ± 0.039 c	0.012 ± 0.001 ab	0.058 ± 0.004 b	0.034 ± 0.003 bcd	0.2 ± 0.0 bc	0.123 ± 0.009 f
Bra6 + Pse5	20.5 ± 1.2 ab	1.7 ± 0.1 c	10.8 ± 0.5 d	6.9 ± 0.3 b	2.9 ± 0.1 bc	2.1 ± 0.1 c	0.111 ± 0.005 d	0.514 ± 0.025 bc	0.009 ± 0.000 d	0.055 ± 0.003 bc	0.033 ± 0.002 cd	0.2 ± 0.0 cd	0.139 ± 0.007 de
Bra6 + Pse6	20.7 ± 2.7 ab	1.8 ± 0.1 c	11.5 ± 0.8 cd	7.0 ± 0.5 b	2.9 ± 0.2 bc	2.3 ± 0.2 c	0.111 ± 0.008 d	0.508 ± 0.036 c	0.012 ± 0.001 a	0.052 ± 0.004 c	0.029 ± 0.002 e	0.3 ± 0.0 a	0.131 ± 0.009 ef
Bra6 + Pse7	19.6 ± 2.2 b	1.9 ± 0.0 bc	11.5 ± 0.3 cd	6.8 ± 0.2 b	2.9 ± 0.1 bc	2.3 ± 0.1 c	0.122 ± 0.003 c	0.538 ± 0.013 bc	0.010 ± 0.000 c	0.058 ± 0.001 b	0.037 ± 0.001 b	0.2 ± 0.0 d	0.150 ± 0.004 cd

† Control: uninoculated treatment. ‡ Data represented as mean (n = 3) ± standard deviation. Different letters in the same column indicate significant differences ($p < 0.05$) in one-way ANOVA.

4. Discussion

The genus *Sinorhizobium* has been found to be a dominant group in nodules of soybean grown in alkaline (pH 7.6–8.0) soils, whereas *Bradyrhizobium* was widely distributed and dominated in neutral to slightly acidic (pH 6.9–7.7) soils [30]. Comparative genomic analysis further revealed that genus-specific genes, known to be involved in alkaline–saline adaptations, likely contributed to the observed biogeographic patterns of these two genera in soybean [31]. Zhang et al. [10] also proposed that the grouping of *Ensifer*- (the heterotypic synonym of *Sinorhizobium*) and *Bradyrhizobium*-dominated clusters from soybean nodules was significantly related to soil pH. Recently, *Bacillus cereus* group was shown to promote and suppress the growth of *Sinorhizobium* and *Bradyrhizobium*, respectively, which affected rhizobial colonization and soybean nodulation under saline–alkali (pH 8) conditions [32]. Bakari et al. [33] demonstrated that soybean inoculation with *Bradyrhizobium* strains effectively increased nodulation and nitrogen fixation in moderately acidic (pH 5.6–5.9) soils. Furthermore, co-application of lime and rhizobial inoculation showed potential to increase nodulation and nitrogen fixation in strongly acidic (pH 4.3–4.8) soils. In this study, the acidotolerant isolate Bra6, which shared high similarity with *Bradyrhizobium diazoefficiens* USDA 110^T in 16S rDNA (99.6%), *nifH* (100%), and *nodD* (99.7%), was recognized as the potential inoculant to induce nodulation of soybean in the strongly acidic (pH 4.06) soils. Previously, several strains belonging to *Bradyrhizobium diazoefficiens* have also been found to nodulate and fix nitrogen when in symbiosis with soybean [34]. Besides, the genetic diversity and distribution of *Bradyrhizobium diazoefficiens* were explored in the rhizosphere of soybean grown in red soil [35].

Despite the fact that *Bradyrhizobium* spp. dominated in soybean nodules, many endophytic bacteria within nodules also showed influences on the soybean–microbe symbiosis and plant growth. *Pseudomonas* has been found as the dominant non-rhizobial group associated with soybean nodules [36]. The synergism between nodule endophyte *Pseudomonas aeruginosa* and *Bradyrhizobium* sp. LSBR-3 was studied, which showed the improvement in plant growth and nutrient acquisition in soybean after dual inoculation [37]. Co-inoculation with *Bacillus thuringiensis* and *Bradyrhizobium japonicum* was demonstrated to enhance soybean nodulation and growth [38,39]. The number and weight of nodules increased, and root weight increased more often than shoot weight under greenhouse and field conditions. Compared with the uninoculated control, we also demonstrated a significant ($p < 0.05$) increment in the biomass of nodules and fresh weight of roots after co-inoculation with *Bradyrhizobium* and *Pseudomonas* isolates in strongly acidic soils. *Pseudomonas* isolates with potentially plant growth-promoting traits showed different influences on rhizobia-mediated symbiotic nitrogen fixation and soybean growth. Most of the *Pseudomonas* species closely related to our isolates have not been reported as soybean-associated bacteria, except that *Pseudomonas glycinae* MS586^T was isolated from the soybean rhizosphere [40]. In this study, the non-rhizobial isolates Pse2, which shared the highest sequence similarity of 16S rDNA with *Pseudomonas punonensis* CECT 8089^T, was demonstrated as the superior partner, showing a synergistic interaction with the rhizobial isolate Bra6.

Zeffa et al. [14] demonstrated that the effects of co-inoculation on plant growth varied according to the PGPR genus used as co-inoculant, as well as with the experimental conditions. Co-inoculation of soybean plants with seed-borne *Bacillus amyloliquefaciens* and *Bradyrhizobium japonicum* significantly improved nodulation, which could be due in part to the production of phytohormones [41]. High levels of IAA produced by nodule endophyte *Bacillus megaterium* or rhizobacterial *Bacillus velezensis* were also considered to aid the development of mature nodules, which thereby improved the nodular nitrogen fixation [42,43]. Defez et al. [44] further demonstrated that IAA-overproducing *Ensifer meliloti* inside nodules increased the activity of nitrogen-fixing apparatus and photosynthetic function. Besides, *Pseudomonas fluorescens* has been found to enhance the nitrogen fixation of soybean through promoting the growth and colonization of *Bradyrhizobium japonicum* [45]. Soybean symbiotic performance was improved by co-inoculation with *Bradyrhizobium japonicum* and *Azospirillum brasilense* through quorum-sensing communi-

cation [46]. In this study, all the *Pseudomonas* isolates showed potential to produce IAA, which might influence the symbiotic outcome by altering phytohormonal homeostasis of soybean [47]. The possibility that metabolites such as siderophores might enhance nodule formation has also been proposed previously [48].

The entrance and survival of the endophytes in roots are generally in relation to their capabilities to hydrolyze plant constituents [49,50]. However, the constitutive release of plant cell wall-degrading enzymes such as pectinase by endophytic bacteria may confer plant pathogenicity [51]. Huang et al. [25] also proposed that endophytes with pectinase activity may be one of the factors contributing to the negative effects on plant growth. In this study, the pectinase activity was recorded in isolates Pse5, Pse6, and Pse7. Besides, the starch, which serves as the storage form of carbohydrates in soybean plant, was only utilized by isolate Bra6 and Pse2. It is assumed that both isolates proliferate using starch instead of pectin as its carbon source, which might be more compatible to the host plant. Moreover, the proteolytic activity was found in all four *Pseudomonas* isolates, except for Pse2. The ability of endophytes to hydrolyze protein might also have an influence on the rhizobium-mediated symbiotic nitrogen fixation, which needs to be clarified in the future.

5. Conclusions

In this study, the effective rhizobial isolates with nodule-inducing capability and non-rhizobial isolates possessing potential plant growth-promoting traits were obtained. Co-inoculation with acidotolerant *Bradyrhizobium* isolate Bra6 and various *Pseudomonas* isolates were demonstrated to enhance nodulation, symbiotic nitrogen fixation, nutrients' acquisition, and biomass of soybeans in strongly acidic soils. This provided insight into the benefits after conducting soybean intercropping as well as bacterial inoculation in the strongly acidic tea-cultivated environments. Furthermore, efforts should be made to elucidate the mechanisms involved in the synergistic association between these isolates, which help to develop effective bioinoculants used to promote soybean growth.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/su132011539/s1, Figure S1: Colony morphology of the five-day bacterial cultures with/without phosphate solubilizing capability on tricalcium phosphate-containing medium. Three replications of each isolate were performed on the same plate. Figure S2: Colony morphology of the five-day bacterial cultures with/without siderophore producing capability on CAS medium. Three replications of each isolate were performed on the same plate. Figure S3: Colony morphology of the five-day bacterial cultures without cellulolytic capability on carboxymethylcellulose-containing medium. Three replications of each isolate were performed on the same plate. Figure S4: Colony morphology of the five-day bacterial cultures with/without pectinolytic capability on pectin-containing medium. Three replications of each isolate were performed on the same plate. Figure S5: Colony morphology of the five-day bacterial cultures with/without amylolytic capability on soluble starch-containing medium. Three replications of each isolate were performed on the same plate. Figure S6: Colony morphology of the five-day bacterial cultures with/without proteolytic capability on skimmed milk-containing medium. Three replications of each isolate were performed on the same plate.

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