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Low-Density Co-Inoculation with *Bradyrhizobium japonicum* SAY3-7 and *Streptomyces griseoflavus* P4 Promotes Plant Growth and Nitrogen Fixation in Soybean Cultivars

Aung Zaw Htwe[1,2], Takeo Yamakawa[1]

[1]Laboratory of Plant Nutrition, Graduate School of Bioresource and Bioenvironmental Sciences, Faculty of Agriculture, Kyushu University, Fukuoka, Japan
[2]Department of Agronomy, Yezin Agricultural University, Yezin, Myanmar
Email: aungzawhtwe333@gmail.com

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Abstract

Inoculation density has a marked effect on nodulation and N fixation in soybean (*Glycine max* L.). Therefore, we conducted this study to determine the optimal inoculation density of *Bradyhizobium japonicum* SAY3-7 (SAY3-7) and *Streptomyces griseoflavus* P4 (P4) for plant growth, nodulation, and N fixation, and to investigate the effect of co-inoculation on selected soybean cultivars, using the optimal inoculation density. Nitrogen fixation, in terms of an acetylene reduction activity value, was measured using a flame ionization gas chromatograph. In this study, low-density single inoculation with P4 (10^5 or 10^6 cells mL^{-1}) was associated with the highest plant biomass, compared with normal- and high-density single inoculation with P4 (10^7 or 10^8 cells mL^{-1}). Moreover, low-density single or co-inoculations with SAY3-7 and/or P4 produced the highest nodule biomass and highest nitrogenase activity, compared with single or dual inoculation at other inoculation densities. Therefore, we evaluated low-density co-inoculation with P4 and SAY3-7, at the rate of 10^5 cells mL^{-1}, on selected soybean cultivars. Low-density co-inoculation increased the plant biomass, compared with un-inoculated plants. The effects of single and co-inoculation on nodulation did not differ significantly for any of the cultivars, except "Yezin-9" in the first experiment and "Shan Seine" in the second experiment. Low-density inoculation with both bacteria increased N fixation by 15% - 75% for seven of the cultivars in the first experiment and by 15% - 39% for three of the cultivars in the second experiment, compared with single inoculation with SAY3-7.

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Based on the overall results, we concluded that low-density co-inoculation with P4 and SAY3-7 gave improved plant growth and N fixation.

Keywords

***Brayrhizobium japonicum* SAY3-7, *Streptomyces griseoflavus* P4, Inoculation Densities, Co-Inoculation, Soybean, Nitrogen Fixation**

1. Introduction

Soybean (*Glycine max* L.), one of the most important leguminous crops in Myanmar, can fix nitrogen (N) from the atmosphere through symbiotic biological N fixation with effective bradyrhizobia. Nitrogen fixation depends on the soybean cultivar, as the compatibility of soybean cultivars differs depending on the inoculated bacteria. Identification of a nodulation regulatory (*Rj*) gene is important for proper inoculation of soybean cultivars, as symbiotic effectiveness, compatibility, and preference of inoculated rhizobia differ among cultivars. Recently, *Rj* genes of Myanmar soybean cultivars were identified based upon inoculation tests and PCR analyses [1] and these included Rj_4, non-*Rj*, Rj_3, and Rj_2Rj_3 genes.

To counter the increasing cost of N fertilizers and the environmental impact caused by N fertilizer application, root nodule bacteria play a role as alternative sources of N. Recently, Htwe *et al*. [2] investigated the diversity of 120 native bradyrhizobia from five different soybean growing regions, using sequence analysis of the 16S-23S rRNA internal transcribed spacer (ITS) region and confirmed that all of the isolates belonged to *Bradyrhizobium*, and most of the isolated strains were Types A or B. An evaluation of the symbiotic effectiveness of 25 *Bradyrhizobium* strains carried out to select the most effective strain [3]. They suggested that *Bradyrhizobium japonicum* SAY3-7 (SAY3-7) fixed higher amounts of N in non-*Rj* and Rj_4 soybean cultivars.

To promote plant growth and reduce the use of chemicals, endophytic bacteria also play a role as plant growth promoters and as bio-control agents. *Streptomyces griseoflavus* P4 (P4) (Accession number: EU741217), isolated from sweet pea root at Kurima, Tsu-City, Japan [4], has 99.7% sequence identity with *S. griseoflavus* [5]. Plant growth-promoting hydrolytic enzymes, such as chitinase [5] and amylase [6], and indole acetic acid (IAA) [7], are produced by P4, which has the ability to inhibit the growth of *Fusarium oxysporum f. sp. Lycopersici* by disrupting the fungal cell wall [5]. Therefore, many researchers recommend co-inoculation of soybean with plant growth promoting endophytic and N-fixing bacteria to increase soybean yield and enhance the sustainability of agriculture [8].

Co-inoculation with P4 and N-fixing rhizobia increases N uptake of adzuki beans and Thai sweet pea [4]. Beneficial effects on N fixation were observed in response to co-inoculation with P4 and indigenous bradyrhizobia in various soybean cultivars such as Yezin-3, Yezin-6, Hinthada, and Shan Sein [9]-[11]. Htwe *et al*. [12] stated that dual inoculation of the Yezin-11 (Rj_4) cultivar with SAY3-7 or *B. elkanii* AHY3-1 and P4 increased N-fixation rates by about 45% and 31%, respectively, and increased shoot growth compared with single inoculation with those strains. Moreover, single inoculation with endophytic P4 promoted plant growth of various leguminous, cereal, and vegetable crops [7] [12] [13].

There are many factors which affect N fixation. To promote N fixation, selection of efficient cultivars and effective strains, and co-inoculation with N-fixing bacteria and compatible plant growth-promoting bacteria are important steps. These steps were assessed in previous studies. In addition, the inoculation rate also has a marked effect on nodulation and N fixation. Albareda *et al*. [14] stated that nodule dry weight, seed yield, and seed N content increased when the number of rhizobia applied to seeds was increased from 10^4 to 10^7 cell $seed^{-1}$. Jitacksorn and Sadowsky [15] stated that nodulation on two soybean cultivars was inhibited when plants were inoculated with a high-density (10^9 cells mL^{-1}) of strain USDA110 grown in complex media. In contrast, more nodules were formed on plants inoculated at a low-density (10^5 cells mL^{-1}). However, determining the proper inoculation density and proper combination of N-fixing bacteria and plant growth-promoting bacteria have not been achieved in Myanmar. Therefore, we conducted this study to determine the proper inoculation density for P4 and SAY3-7, and to test the single or co-inoculation effects on plant growth and N fixation of various soybean cultivars.

2. Materials and Methods

2.1. Inoculum Preparation

SAY3-7 was previously isolated from a soybean nodule and identified by Htwe *et al*. [2]. P4 was isolated from a sweet pea root by Thapanapongworakul [4] and identified by Tang-um and Niamsup [5]. The bradyrhizobial strain SAY3-7, was cultured in A1E liquid medium [16] and incubated on a rotary shaker at 30˚C for 7 days. P4 was cultured on Inhibitory Mold Agar (IMA)-2 liquid medium [17] and incubated on a rotary shaker (100 rpm) at 30˚C for 5 days.

Liquid bacterial suspensions of both bacteria were diluted with half-strength Modified Hoagland's Nutrient (MHN) solution [18] to prepare the required inoculation density. Treatments were single inoculations at a density ranging from 10^5 to 10^8 cells mL^{-1} for both bacteria individually and for combinations of different inoculation densities of SAY3-7 and P4. According to the reports of Jitacksorn and Sadowsky [15] and Lohrke *et al*. [19], 10^5 rhizobial cells mL^{-1} and 10^8 rhizobial cells mL^{-1} were used as low-inoculation density and high-inoculation density, respectively, in our experiment.

2.2. Cultivation

To determine the proper inoculation density for both bacteria, Yezin-11 (Rj_4) was used as a host plant. After determining the proper combination of inoculant densities (P4, 10^5 cells mL^{-1};SAY3-7, 10^5 cells mL^{-1}), single or dual inoculations of these bacteria were tested on 10 soybean cultivars: Yezin-3 (Rj_4), Yezin-6 (*non-Rj*), Yezin-8 (*non-Rj*), Yezin-9 (Rj_3), Yezin-10 (Rj_2Rj_3), Yezin-11 (Rj_4), Shan Seine [local] (Rj_4), Hinthada (local, Rj_4), and Madaya (local, Rj_4). Yezin-3 (Rj_4), Yezin-6 (*non-Rj*), Yezin-8 (*non-Rj*), Yezin-9 (Rj_3), Yezin-10 (Rj_2Rj_3), Yezin-11 (Rj_4) were produced by Department of Agricultural Research, Yezin, Myanmar. These cultivars were recommended for farmers as high yielding cultivars. These soybean cultivars were obtained from the Food Legume Section, Department of Agricultural Research, Yezin, Myanmar. Nodulation regulatory genes (*Rj*) of these cultivars were identified by Soe *et al*. [20] and Htwe *et al*. [1]. Surface sterilization of seeds was performed as described in a previous report [2]. One-liter pots were filled with vermiculite and 0.6 L of half-strength modified MHN solution. The pots were autoclaved at 120˚C for 20 min. Each seed was inoculated with 5 mL of bacterial suspension. The plants were cultivated in an environmentally controlled room (25˚C and 75% relative humidity) for 4 weeks. Watering was performed as necessary. Autoclaved deionized water was used in this study. This study was conducted from December 2015 to March 2016. The pots were randomly assigned for each cultivar.

2.3. Data Collection

For nitrogenase activity measurements, soybean plants were cut at the cotyledonary nodes and roots, with intact nodules placed in 100-mL conical flasks. The flasks were sealed with a serum stopper and injected with 12 mL of acetylene (C_2H_2) gas to replace the air. Subsamples (1 mL) were analyzed for ethylene (C_2H_4) concentration at 5 and 65 min after injecting with C_2H_2 gas using a flame ionization gas chromatograph (GC-14A, Shimadzu, Kyoto, Japan) equipped with a stainless steel column (3 mm diameter and 0.5 m length) as previously described [10]. After the assay, nodules were counted. Shoots, roots, and nodules were collected separately and oven dried at 70˚C for 24 hours to record dry weights. Data were recorded from three replicates.

2.4. Data Analysis

Data were analyzed using the STATISTIX 8 software package (Analytical Software, Tallahassee, FL, USA) and the means were compared by Tukey's HSD test with a P value < 0.05 to test for statistical significance.

3. Results (Tables 1-3)

3.1. Effects of Inoculation Density on Plant Growth, Nodulation, and Nitrogen Fixation

In this study, single inoculation with various rates of the N-fixing bacterium SAY3-7 and the plant growth-promoting endophytic bacterium P4, and co-inoculation using various combinations of both bacteria, were tested for effects on plant growth, nodulation, and N fixation. Shoot dry biomasses did not differ significantly among treatments. However, single or co-inoculation with P4 and/or SAY3-7 resulted in higher shoot biomasses compared

Table 1. Effect of different inoculation densities of *B. japonicum* SAY3-7 and *S. griseoflavus* P4 plant growth, nodulation and acetylene reduction activity of Yezin-11 soybean cultivar at 28 DAS.

Treatment	SDW ($g \cdot plant^{-1}$)	RDW ($g \cdot plant^{-1}$)	NN (No. $plant^{-1}$)	NDW ($mg \cdot plant^{-1}$)	ARA ($\mu mol\ C_2H_4\ h^{-1} \cdot plant^{-1}$)
Control	0.28 a	0.11 a	0.00 c	0.00 b	0.00 b
P4 10^5	0.31 a	0.14 a	0.00 c	0.00 b	0.00 b
P4 10^6	0.30 a	0.15 a	0.00 c	0.00 b	0.00 b
P4 10^7	0.27 a	0.14 a	0.00 c	0.00 b	0.00 b
P4 10^8	0.26 a	0.12 a	0.00 c	0.00 b	0.00 b
SAY3-7 10^5	0.30 a	0.12 a	7.00 ab	21.33 a	0.71 ab
SAY3-7 10^5 + P4 10^5	0.29 a	0.13 a	3.67 abc	15.80 a	0.92 a
SAY3-7 10^5 + P4 10^6	0.34 a	0.14 a	8.67 ab	17.13 a	0.47 ab
SAY3-7$10^5$ + P4 10^7	0.30 a	0.14 a	4.00 abc	14.50 ab	0.65 ab
SAY3-7 10^5 + P4 10^8	0.35 a	0.12 a	6.00 abc	17.20 a	0.86 a
SAY3-7 10^6	0.30 a	0.13 a	4.67 abc	14.93 a	0.71 ab
SAY3-7 10^6 + P4 10^5	0.34 a	0.12 a	7.00 ab	19.33 a	0.76 a
SAY3-7 10^6 + P4 10^6	0.30 a	0.14 a	6.00 abc	15.70 a	0.48 ab
SAY3-7 10^6 + P4 10^7	0.30 a	0.12 a	5.67 abc	14.00 ab	0.62 ab
SAY3-7 10^6 + P4 10^8	0.31 a	0.13 a	6.00 abc	16.17 a	0.58 ab
SAY3-7 10^7	0.33 a	0.14 a	3.33 bc	12.00 ab	0.45 ab
SAY3-7$10^7$ + P4 10^5	0.31 a	0.12 a	3.33 bc	9.73 ab	0.37 ab
SAY3-7 10^7 + P4 10^6	0.33 a	0.14 a	7.67 ab	18.27 a	0.57 ab
SAY3-7 10^7 + P4 10^7	0.32 a	0.15 a	10.00 ab	18.50 a	0.25 ab
SAY3-7$10^7$ + P4 10^8	0.35 a	0.14 a	5.67 abc	14.67 ab	0.54 ab
SAY3-7 10^8	0.32 a	0.13 a	8.33 ab	14.03 ab	0.52 ab
SAY3-7 10^8 + P4 10^5	0.28 a	0.10 a	6.33 abc	9.67 ab	0.40 ab
SAY3-7$10^8$ + P4 10^6	0.29 a	0.11 a	4.00 abc	11.43 ab	0.56 ab
SAY3-7 10^8 + P4 10^7	0.26 a	0.10 a	10.33 a	13.90 ab	0.44 ab
SAY3-7 10^8 + P4 10^8	0.36 a	0.15 a	7.67 ab	19.37 a	0.60 ab

Mean values in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). NN; nodule number; NDW; nodule dry weight; SDW; shoot dry weight; RDW; root dry weight; ARA; acetylene reduction activity.

Table 2. Effect of low density inoculation of *B. japonicum* SAY3-7 and *S. griseoflavus* plant growth, nodulation and acetylene reduction activity of different soybean varieties at 28 DAS.

Cultivar	Treatment	SDW ($g \cdot plant^{-1}$)	RDW ($g \cdot plant^{-1}$)	NN (No. $plant^{-1}$)	NDW ($mg \cdot plant^{-1}$)	ARA ($\mu mol\ C_2H_4\ h^{-1} \cdot plant^{-1}$)
Yezin-3	Control	0.16 a	0.07 a	0.00 b	0.00 b	0.00 a
	P4 10^5	0.19 a	0.08 a	0.00 b	0.00 b	0.00 a
	SAY3-7 10^5	0.21 a	0.09 a	18.33 a	13.40 a	0.35 a
	SAY3-7 10^5 + P4 10^5	0.21 a	0.09 a	16.00 a	13.53 a	0.60 a

Continued

Yezin-6	Control	0.20 b	0.08 b	0.00 b	0.00 b	0.00 b
	P4 10^5	0.27 ab	0.12 ab	0.00 b	0.00 b	0.00 b
	SAY3-7 10^5	0.32 a	0.14 a	3.33 a	12.20 a	0.91 a
	SAY3-7 10^5 + P4 10^5	0.32 a	0.15 a	4.00 a	12.03 a	0.55 ab
Yezin-7	Control	0.17 a	0.05 ab	0.00 b	0.00 b	0.00 b
	P4 10^5	0.15 a	0.06 a	0.00 b	0.00 b	0.00 b
	SAY3-7 10^5	0.16 a	0.05 a	8.00 a	8.77 a	0.33 a
	SAY3-7 10^5 + P4 10^5	0.15 a	0.05 a	5.67 a	5.37 a	0.15 ab
Yezin-8	Control	0.24 a	0.10 a	0.00 b	0.00 b	0.00 b
	P4 10^5	0.29 a	0.12 a	0.00 b	0.00 b	0.00 b
	SAY3-7 10^5	0.27 a	0.11 a	8.00 a	18.63 a	0.95 a
	SAY3-7 10^5 + P4 10^5	0.28 a	0.11 a	8.67 a	13.83 a	0.65 a
Yezin-9	Control	0.18 a	0.07 b	0.00 c	0.00 c	0.00 b
	P4 10^5	0.21 a	0.09 ab	0.00 c	0.00 c	0.00 b
	SAY3-7 10^5	0.17 a	0.08 ab	10.33 b	3.60 b	0.06 ab
	SAY3-7 10^5 + P4 10^5	0.24 a	0.10 a	18.00 a	8.37 a	0.12 a
Yezin-10	Control	0.20 a	0.08 a	0.00 b	0.00 b	0.00 a
	P4 10^5	0.22 a	0.09 a	0.00 b	0.00 b	0.00 a
	SAY3-7 10^5	0.23 a	0.08 a	5.67 a	3.97 a	0.22 a
	SAY3-7 10^5 + P4 10^5	0.23 a	0.09 a	4.67 ab	3.37 ab	0.30 a
Yezin-11	Control	0.21 b	0.08 b	0.00 b	0.00 b	0.00 c
	P4 10^5	0.35 a	0.18 a	0.00 b	0.00 b	0.00 c
	SAY3-7 10^5	0.32 a	0.15 a	6.67 a	14.73 a	0.34 b
	SAY3-7 10^5 + P4 10^5	0.27 ab	0.13 ab	7.00 a	12.00 a	0.68 a
Shan Seine	Control	0.21 a	0.08 a	0.00 b	0.00 b	0.00 a
	P4 10^5	0.24 a	0.09 a	0.00 b	0.00 b	0.00 a
	SAY3-7 10^5	0.24 a	0.06 a	10.00 a	17.80 a	0.57 a
	SAY3-7 10^5 + P4 10^5	0.25 a	0.08 a	10.00 a	17.20 a	0.67 a
Hinthada	Control	0.20 b	0.07 a	0.00 b	0.00 b	0.00 c
	P4 10^5	0.21 ab	0.07 a	0.00 b	0.00 b	0.00 c
	SAY3-7 10^5	0.22 ab	0.06 a	13.00 a	6.17 a	0.06 b
	SAY3-7 10^5 + P4 10^5	0.25 a	0.08 a	12.67 a	8.40 a	0.24 a
Madaya	Control	0.11 a	0.03 b	0.00 b	0.00 b	0.00 b
	P4 10^5	0.11 a	0.05 a	0.00 b	0.00 b	0.00 b
	SAY3-710^5	0.11 a	0.04 ab	5.33 a	3.87 a	0.12 ab
	SAY3-7 10^5 + P4 10^5	0.08 a	0.04 ab	3.33 ab	5.53 a	0.28 a

Mean values for each variety in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). NN; nodule number; NDW; nodule dry weight; SDW; shoot dry weight; RDW; root dry weight; ARA; acetylene reduction activity. Parallel tests were not done in this experiment.

Table 3. Effect of low density inoculation of *B. japonicum* SAY3-7 and *S. griseoflavus* P4 on plant growth, nodulation and acetylene reduction activity of selected soybean varieties at 28 DAS.

Cultivar	Treatment	SDW ($g \cdot plant^{-1}$)	RDW ($g \cdot plant^{-1}$)	NN (No. $plant^{-1}$)	NDW ($mg \cdot plant^{-1}$)	ARA ($\mu mol\ C_2H_4\ h^{-1} \cdot plant^{-1}$)
Yezin-6	Control	0.38 a	0.16 ab	0.00 b	0.00 b	0.00 a
	P4 10^5	0.40 a	0.19 a	0.00 b	0.00 b	0.00 a
	SAY3-7 10^5	0.35 a	0.14 b	6.83 a	14.35 a	0.28 a
	SAY3-7 10^5 + P4 10^5	0.43 a	0.14 b	5.33 a	21.08 a	0.42 a
Yezin-8	Control	0.32 b	0.15 ab	0.00 c	0.00 b	0.00 b
	P4 10^5	0.42 a	0.17 a	0.00 c	0.00 b	0.00 b
	SAY3-7 10^5	0.30 b	0.12 c	11.00 b	16.12 a	0.31 a
	SAY3-7 10^5 + P4 10^5	0.39 a	0.14bc	14.00 a	15.28 a	0.16 ab
Yezin-11	Control	0.36 a	0.16 a	0.00 b	0.00 b	0.00 b
	P4 10^5	0.39 a	0.20 a	0.00 b	0.00 b	0.00 b
	SAY3-7 10^5	0.38 a	0.17 a	4.83 a	11.08 a	0.25 ab
	SAY3-7$10^5$ + P4 10^5	0.41 a	0.18 a	4.50 a	14.97 a	0.41 a
Shan Seine	Control	0.32 b	0.13 ab	0.00 c	0.00 c	0.00 c
	P4 10^5	0.34 b	0.15 a	0.00 c	0.00 c	0.00 c
	SAY3-7 10^5	0.33 b	0.10 c	10.50 b	18.47 b	0.31 b
	SAY3-7 10^5 + P4 10^5	0.41 a	0.12bc	14.17 a	27.85 a	0.42 a

Mean values for each variety in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). NN; nodule number; NDW; nodule dry weight; SDW; shoot dry weight; RDW; root dry weight; ARA; acetylene reduction activity. Parallel tests were not done in this experiment.

with the control, except for high density inoculation with P4 (10^7 and 10^8 cells mL^{-1}) and the co-inoculation treatment (SAY3-7 at 10^8 cells mL^{-1} and P4 at 10^7 cells mL^{-1}). Similarly, higher root dry biomasses were also obtained from single or co-inoculation with P4 and/or SAY3-7, except for some treatments. There were significant differences in nodulation, based on nodule number and nodule dry weight, among the control, P4, and single or dual inoculations with SAY3-7 and/or P4, as P4 alone did not form nodules. The highest nodule numbers were observed from co-inoculation (SAY3-7 at 10^8 cells mL^{-1} and P4 at 10^7 cells mL^{-1}), but they did not differ significantly from those of plants inoculated with SAY3-7. Higher nodule dry weight was obtained from a single inoculation with SAY3-7 (10^5 cells mL^{-1}), but it did not differ significantly from other inoculation treatments with SAY3-7. N fixation, in terms of the acetylene reduction assay (ARA) value, differed significantly among the control, P4, and single or dual inoculations with SAY3-7 and/or P4, as P4 alone had nonitrogenase activity. The highest N-fixing potential was observed with SAY3-7 at 10^5 cells mL^{-1} and P4 at 10^5 cells mL^{-1}, followed by SAY3-7 at 10^5 cell mL^{-1} and P4 at 10^8 cell mL^{-1}, and SAY3-7 at 10^6 cells mL^{-1} and P4 at 10^5 cell mL^{-1}. Single inoculations of SAY3-7, at 10^5 and 10^6 cells mL^{-1}, produced the fourth highest values of nitrogenase activity. Based on these results, we used low-density co-inoculation (SAY3-7 at 10^5 cells mL^{-1} and P4 at 10^5 cells mL^{-1}) for further study because it yielded the highest nitrogenase activity. Moreover, single inoculation with SAY3-7 (10^5 cells mL^{-1}) produced the highest nodule biomass. Single inoculation with P4 (10^5 or 10^6 cells mL^{-1}) resulted in the highest plant biomass compared with normal and high density (10^7 or 10^8 cells mL^{-1}, respectively) single inoculations.

3.2. Effects of Single or Co-Inoculation with SAY3-7 on Plant Growth, Nodulation, and Nitrogen Fixation in Selected Soybean Cultivars

For Yezin-3, there were no significant differences in shoot or root dry weights. Nitrogenase activity also did not differ significantly between single and co-inoculations. However, co-inoculation increased N fixation. Single or co-inoculation with SAY3-7 increased shoot and root biomasses significantly. Single inoculation with SAY3-7 resulted in the highest nitrogenase activity, but it did not differ significantly from co-inoculation and had repressive effects on N fixation in co-inoculated plants.

For Yezin-7, there were no significant differences in shoot or root dry weights. Single or co-inoculation did not differ significantly in terms of nodulation. Single inoculation with SAY3-7 achieved the highest nitrogenase activity, but the activity was not significantly different from that produced by co-inoculation. Repressive effects on N fixation were observed in co-inoculated plants.

For Yezin-8, there were no significant differences in shoot or root dry weights. Single inoculation with SAY3-7 resulted in the highest nitrogenase activity, but the activity did not differ significantly from that produced by co-inoculation. Repressive effects on N fixation occurred in co-inoculated plants.

For Yezin-9, there were no significant differences in shoot dry weight. Significant differences in root dry weight were observed for the co-inoculation treatment, although there was no significant difference between single inoculations with SAY3-7 and P4. Nitrogenase activity also did not differ significantly between the single and co-inoculation methods. Co-inoculation increased N fixation by approximately 50%, compared with single inoculation with SAY3-7.

For Yezin-10, there were no significant differences in shoot or root dry weight. Single and co-inoculations were not significantly different in terms of nodulation. Nitrogenase activity also did not differ significantly between the single and co-inoculation treatments. However, co-inoculation increased N fixation by approximately 40%, compared with single inoculation with SAY3-7.

For Yezin-11, shoot and root dry weights of inoculated plants differed significantly from those of the control plants. Single inoculation with P4 produced the highest shoot and root dry weights. Nitrogenase activity differed significantly between the single and co-inoculation treatments. Co-inoculation increased N fixation by approximately 50% compared with single inoculation with SAY3-7.

For Shan Seine, there were no significant differences in shoot or root dry weights. Nitrogenase activity also did not differ significantly between the single and co-inoculation treatments. However, co-inoculation increased N fixation by approximately 15% compared with single inoculation.

For Hinthada, the shoot dry weight of the inoculated plants differed significantly from that of control plants. Co-inoculation with SAY3-7 and P4 produced the highest shoot dry weight. Moreover, co-inoculation increased root dry weight compared with the single inoculation and control treatments, although differences were not significant. Nitrogenase activity differed significantly when comparing the single and co-inoculation treatments. Co-inoculation increased N fixation by approximately 75% compared with single inoculation with SAY3-7.

For Madaya, shoot dry weights did not differ significantly among the treatments. However, the root dry weights of inoculated plants differed significantly from those of the control plants. Single inoculation with P4 produced the highest root dry weight. Nitrogenase activity differed significantly between the single and co-inoculation treatments. Co-inoculation increased N fixation by approximately 57% compared with single inoculation with SAY3-7.

In all cultivars, significant differences were observed in nodulation, as control plants and plants inoculated with P4 could not from nodules. However, single and co-inoculation did not differ significantly with respect to nodulation for all cultivars, with the exception of Yezin-9.

3.3. Effects of Single or Co-Inoculation with P4 on Plant Growth, Nodulation, and Nitrogen Fixation in Selected Soybean Cultivars

For Yezin-6, shoot growth and nodulation did not increase significantly among single and co-inoculation treatments with SAY3-7 and the control. However, root dry weight produced by P4 single inoculation was significantly higher than that of other treatments. Nitrogenase activity did not differ significantly among treatments; however, co-inoculation increased N fixation by approximately 15% compared with single inoculation with SAY3-7.

For Yezin-8, differences in shoot and root dry weights were significant. Single inoculation with P4 resulted in the highest shoot biomass, but it did not differ significantly from that obtained with co-inoculation. Moreover, single inoculation with P4 also produced the highest root biomass, although it did not differ significantly from that of the control. A significantly higher number of nodules was obtained from co-inoculation with SAY3-7 and

P4, whereas nodule dry weight did not differ significantly when comparing single and dual inoculation treatments. Single inoculation with SAY3-7 produced the highest nitrogenase activity and had a depressive effect on N fixation was observed in co-inoculated plants.

For Yezin-11, shoot and root dry weights did not differ significantly among treatments. Significant differences were observed for nodulation, as control plants and plants inoculated with P4 could not form nodules. However, single and co-inoculation did not differ significantly with respect to nodulation. Nitrogenase activity did not different significantly between the single and co-inoculation treatments. Co-inoculation increased N fixation by approximately 39% compared with single inoculation with SAY3-7.

For Shan Seine, differences in shoot and root dry weights were significant. Single inoculation with P4 produced significantly higher shoot dry weight. The significantly higher root dry weight was obtained from the co-inoculation treatment, but it was not different from the control. Significantly higher numbers of nodules and nodule dry weight were observed in co-inoculated plants. Moreover, co-inoculation increased N fixation significantly by approximately 26% compared with single inoculation with SAY3-7.

4. Discussion

Inoculation is used to provide sufficient, effective, and viable rhizobia to enhance rapid colonization in the rhizosphere. Nodules are formed soon after germination, to produce optimum yields [21] [22]. Inoculation of soybean with the normal dose of efficient rhizobia does not improve seed yield significantly. The occupancy of inoculated strains in nodules is relatively low compared with less efficient indigenous rhizobia due to their competition to occupy root nodules [23] [24]. However, suppression of nodulation was observed in high- and low-nodulating cultivars, probably due to high inoculation density [25]. In our study, nodulation and N fixation at higher inoculation densities (10^7 and 10^8 cells mL^{-1}) were low compared with those at low inoculation densities (10^5 and 10^6 cells mL^{-1}). Our findings support the findings of Yamakawa and Fukushima [26], in which the application of a higher inoculum density decreased acetylene reduction activity. There was also a significant increment in yield increase observed at inoculation rates of 10^5 and 10^7 cells $seed^{-1}$. In our study, the highest N fixation was obtained from low-density co-inoculation with SAY3-7 at 10^5 cells mL^{-1} and P4 at 10^5 cells mL^{-1}, followed by co-inoculation with SAY3-7 at 10^5 cells mL^{-1} and P4 at 10^8 cells mL^{-1}, and SAY3-7 at 10^6 cells mL^{-1} and P4 at 10^5 cells mL^{-1}. Recently, Soe *et al.* [11] reported significant increases in biomass production, the relative ureide index, percentage of N derived from atmospheric N, and seed yield in Yezin-6 under open field conditions at the Kyushu University Farm, Japan. These responses were due to low density co-inoculation with P4 and *B. yuanmingense* MAS34 (10^5 cells $seed^{-1}$). According to the results from this study, low density co-inoculations of Yezin-11 with P4 and SAY3-7 (10^5 cells mL^{-1}) were very effective in terms of nodulation, N fixation, and plant growth. We showed that low inoculation density was optimal for soybean. It has been reported that 10^5 - 10^6 rhizobia per seed are required to obtain maximum soybean seed yields [27] [28].

In this study, shoot, and/or root biomass were increased in some soybean cultivars in response to single or co-inoculation with P4. A previous study [12], showed that shoot and root biomasses of soybean cultivars were increased due to inoculation with P4. It has been reported that root and shoot biomasses were increased significantly in plants inoculated with P4, compared with un-inoculated plants [7] [13] [29]. Moreover, incremental increases in shoot and root biomass of cereal crops such as maize (*Zea mays* L.) and rice (*Oryza sativa* L.), and vegetables such as radish, spinach, and komatsuna, were also reported [7] [12]. In this study, co-inoculation with P4 with SAY3-7 increased N fixation of soybean cultivars harboring Rj_4-genes by approximately 15% - 75%. Our findings are in agreement with previous reports, in which co-inoculation with P4 increased N fixationsignificantly in Yezin-11 (Rj_4) by approximately 41% [12]. Moreover, this result supported the findings by others that co-inoculation with a bradyrhizobial strain and an endophytic bacterium P4 increases N fixation in various soybean cultivars [4] [10] [11] [20]. Co-inoculation of soybean with SAY3-7 and P4 showed detrimental effects on N fixation rates in Yezin-6 (non-*Rj*) and Yezin-8 (non-*Rj*) in the first experiment, and in Yezin-8 (non-*Rj*) in the second experiment compared with single inoculation with these bacteria. Soe and Yamakawa [11] highlighted that co-inoculation with P4 and *B. yuanmingense* showed beneficial effects on plant growth and N fixation in Yezin-6 (non-*Rj*). In previous studies, co-inoculation with P4 and SAY3-7 had no repressive effect on N fixation in Yezin-6 (non-*Rj*) [12]. Moreover, Htwe *et al.* [12] previously reported that co-inoculation with SAY3-7 or *B. elkanii* AHY3-1 and P4 might be useful for bio-fertilizer production to fulfill the requirements for nitrogenous fertilizer by enhancing plant growth and N fixation in soybean. Our study also highlighted that co-inoculation with SAY3-7 and P4 could be used to produce bio-fertilizer, because low-density co-inoculation with SAY3-7 and P4 promoted plant growth and N fixation in various soybean cultivars.

5. Conclusion

In this study, single or co-inoculation with P4 and/or SAY3-7, at the rate of 10^5 or 10^6 cells mL^{-1} resulted in increased nitrogenase activity, nodule mass, and plant biomass compared with single or co-inoculation at higher inoculation densities. Moreover, low density co-inoculation (SAY3-7 at 10^5 cells mL^{-1} and P4 at 10^5 cells mL^{-1}) increased the plant biomass of soybean cultivars compared with un-inoculated plants. Although nodulation did not differ significantly in response to single or co-inoculation for most cultivars, low-density co-inoculation with both bacteria increased N fixation by 15% - 75%. Both experiments compared single and co-inoculations with SAY3-7. A depressive effect on N fixation occurred in some cultivars in response to co-inoculation with P4, but most of the cultivars exhibited positive effects on N fixation. According to our overall results, it can be concluded that low-density co-inoculation with P4 and SAY3-7 provides increase efficacy for plant growth and N fixation. This study was conducted under environmentally controlled conditions. To obtain more reliable data, field experiment using low inoculation density should be done as a further study.

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