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Low-Density Co-Inoculation of Myanmar *Bradyrhizobium yuanmingense* MAS34 and *Streptomyces griseoflavus* P4 to Enhance Symbiosis and Seed Yield in Soybean Varieties

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ABSTRACT

This study examined whether low-density co-inoculation of Myanmar *Bradyrhizobium yuanmingense* strain MAS34 and *Streptomyces griseoflavus* P4 would enhance nodulation, N_2 fixation, and seed yield in two soybean varieties. A field experiment was conducted during the July to November 2012 growing season at Kyushu University Farm, Japan, using a split-plot design with three replications and the following four treatments: T1, an uninoculated treatment with peat moss (uninoculated); T2, a single inoculation with *S. griseoflavus* P4 (P4); T3, a single inoculation of *B. yuanmingense* MAS34 (MAS34); and T4, a dual inoculation of P4 with MAS34 (P4 + MAS34). Two varieties of soybean, Yezin-3 (Rj_4) and Yezin-6 (non-*Rj*), were used. The N_2 fixation activity of soybean was evaluated by the relative ureide method using xylem solute from root bled sap at the early pod-fill stage (R3.5). Dry matter production, N_2 fixation, and seed yield were significantly ($P < 0.01$) different between the inoculated treatments. The effect of variety was also significant ($P < 0.05$) for nodule dry weight at the V6 stage, percentage of N derived from the atmosphere at the R3.5 stage, and seed yield at the maturity stage. The number of nodules on the tap roots was significantly higher in Yezin-3 than in Yezin-6. The single inoculation of P4 did not have a significant effect on dry matter production, N_2 fixation, and seed yield in either soybean variety. The dry matter production, relative ureide index, percentage of N derived from the atmosphere, and seed yield were significantly ($P < 0.01$) enhanced by a single inoculation of MAS34 in Yezin-3 and by dual inoculation of P4 + MAS34 in Yezin-6. These results indicate that low inoculum concentrations (10^5 cells seed^{-1}) increase N_2 fixation and seed yield in these soybean varieties under open field conditions. Myanmar *B. yuanmingense* MAS34 and *S. griseoflavus* P4 are expected to be useful biofertilizers for soybean production.

Keywords: *Bradyrhizobium yuanmingense*; N_2 Fixation; S. Griseoflavus P4; Seed Yield; Soybean

1. Introduction

Soybean (*Glycine max* L. Merr.) is the most important grain legume crop in the world and an important protein source for both humans and livestock [1]. In Myanmar, soybean has been cultivated for centuries, providing not only a nutritious food, but also helping to increase the soil fertility [2]. With demands for this legume increasing domestically and abroad, soybean has become the second largest crop cultivated in Myanmar [3].

Soybean helps to maintain soil fertility by assimilating nitrogen from the atmosphere through symbiotic biological N_2 fixation (BNF) with bradyhizobia [4]. BNF is of agronomic importance because it reduces the need for chemical nitrogen fertilizer [5], and nitrogen fixation in the root nodule is thus an important area of study. The level of nitrogen fixation varies by cultivar in many legume crop species. Some crops, particularly combinations of strains and cultivars, have been shown to be especially efficient at N_2 fixation [6]. Several studies have also reported differences between *Bradyrhizobium* strains regarding their effectiveness with different soybean genotypes [7-9].

Weaver and Frederick [10] reported that to successfully compete with indigenous rhizobium, the introduced

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inoculum must be 1000 times greater. However, in Canada, the standard inoculum for soybean, kidney bean, and pea is applied at 10^5 cells·seed^{-1} [11]. Furthermore, Yamakawa and Saeki [12] conducted a field experiment at Kyushu University Farm in Japan to examine the effect of inoculum density and the use of different inoculation methods on soybean production. They found that a higher inoculum density decreased acetylene reduction activity. Significantly increased yield was found at inoculation concentrations of 10^5 cell·seed^{-1} and 10^7 cell·seed^{-1}, but there was no effect of increasing the inoculum above the latter density.

Streptomyces griseoflavus P4 was identified by a full 16S rRNA sequencing, and was mostly related to *S. griseoflavus* (99.7% identical score) with the GenBank database accession number of JN102356 [13]. Significant stimulation effects were observed following the dual inoculation of P4 and the *Bradyrhizobium* strain on N uptake in adzuki bean, Thai sweet pea [14], and soybean [15] compared to a bradyrhizobial single inoculation.

Many studies have determined the dry weight and nitrogen fixation efficiency of indigenous rhizobial strains in leguminous plants [16-18]. Our previous laboratory study revealed that the dual inoculation of *S. griseoflavus* P4 with Myanmar *B. yuanmingense* MAS34 significantly influenced the nodule dry weight in Yezin-6 (non-*Rj*) soybean and the nodule dry weight and N_2 fixation in Yezin-3 (Rj_4) soybean [15]. The experimental data suggested the usefulness of further field investigations of selected *S. griseoflavus* P4 and indigenous *B. yuanmingense* MAS34 with Yezin-3 (Rj_4) and Yezin-6 (non-*Rj*) soybean varieties. This study was designed to verify the previous finding and determine whether plant growth and nitrogen fixation ability were improved in these two soybean varieties under field conditions. Specifically, the main objective of this study was to evaluate the co-inoculation of Myanmar *B. yuanmingense* MAS34 and *S. griseoflavus* P4 to enhance symbiosis and seed yield in two soybean varieties, using a low density of inoculum (10^5 cells·seed^{-1}).

2. Materials and Methods

2.1. Experimental Site

The experiment was conducted at Kyushu University Farm, Fukuoka Prefecture, Japan (33˚37'N, 130˚25'E), during the July to November 2012 growing season. The cultivated field had a clay loam soil containing kaolinite with a pH ranging from 6.1 to 6.4 (soil:water, 1:2.5) or 5.0 to 5.3 (soil:KCl, 1:2.5). The cation exchange capacity (CEC) of the soil was 20.20 $cmol_c·kg^{-1}$ [19]. The soil had a total nitrogen (N) content of 117 mg per 100 g of soil [20], total phosphorus (P) content of 76.5 mg per 100 g of soil [21], available phosphorus (P) content of 9.00 mg per 100 g of soil [22], and exchangeable potassium (K), calcium (Ca), and magnesium (Mg) contents of 0.94, 6.31, and 1.69 $cmol_c·kg^{-1}$, respectively [19]. To estimate the density of indigenous rhizobia, soil samples at 15 cm depth were collected from six locations in the experimental field before fertilization and the rhizobia density was measured by the most probable number (MPN) method [23] using two soybean cultivars: Yezin-3 (Rj_4-genotype) and Yezin-6 (non-*Rj*-genotype).

2.2. Plant Materials

The soybean cultivars Yezin-3 (Rj_4) and Yezin-6 (non-*Rj*) [24] were collected from the Food Legumes Section, Department of Agricultural Research, Yezin, Myanmar. Rj_4 and non-*Rj* in parentheses indicate the nodulation regulatory genes [25]. The *Rj* genes play a role in controlling a plant's compatibility with specific rhizobial strains and also the preference for indigenous soybean-nodulating rhizobia [26,27]. In addition, non-*Rj* is compatible with all bradyrhizobial strains, but Rj_4 has unique features that restrict nodulation with specific strains of *Bradyrhizobium* [28]. Yezin-3 (Rj_4) and Yezin-6 (non-*Rj*) are well-known soybean cultivars in Myanmar.

2.3. Bacteria Maintenance

The *B. yuanmingense* MAS34 was stored at −85˚C on HM salts glycerol stock. It was cultured in A1E liquid media [29] on a rotary shaker (100 rpm) at 30˚C for seven days. The stock culture of *S. griseoflavus* P4 was stored at 4˚C in IMA-2 medium [30] and cultured in a broth medium on a rotary shaker (100 rpm) at 30˚C for five days.

2.4. Land Preparation, Inoculation, and Planting

Prior to planting the field was prepared according to conventional practices. Chemical fertilizers containing urea, super phosphate, and potassium chloride (30.87, 308.75, and 61.75 kg·ha^{-1}) were applied at the rate recommended by the Department of Agricultural Research, Ministry of Agriculture and Irrigation, Myanmar. The land was then leveled and divided into individual plots. The site was 15 m long and 24 m wide. The row width and intra-row spacing were 60 and 20 cm, respectively.

Peat moss inoculum for 100 soybean seeds was prepared from 1.5 mL deionized water and 10 mL of 12% aqueous solution of gum Arabic, 10 g of BM2 (raw materials: peat moss, Group Berger Peat Moss Ltd., Canada), 0.01 mL of 1×10^9 cell·mL^{-1} of *B. yuanmingense* MAS 34 (1×10^5 cells seed^{-1}), and 1 mL of 1×10^7 cell·mL^{-1} of *S. griseoflavus* P4 (1×10^5 cells·seed^{-1}). All inoculation occurred just before planting. Seeds were allowed to mix well with the peat moss inoculum prior to seed sowing.

Three inoculated soybean seeds per hill of peat were sown in the field and immediately covered with rice ash. A split plot design was used with three replications and the following four treatments: T1, an uninoculated treatment with peat moss (uninoculated); T2, a single inoculation with *S. griseoflavus* P4 (P4), T3, a single inoculation of *B. yuanmingense* MAS34 (MAS34); and T4, a dual inoculation of P4 with MAS34 (P4 + MAS34). At the two trifoliate stage (V2 stage), the seedlings were thinned to two seedlings per hill, and inter-cultivation and manual weeding were undertaken. Some pesticides and insecticides were periodically applied when pests and insects were observed.

2.5. Plant Sampling

Plant samples were collected from three growing stages: the six unfolded trifoliate leaves stage (V6 stage), early pod-fill stage (R3.5 stage), and the maturity stage. For the V6 and R3.5 stages, two plants in one hill per plot were sampled and separated into nodules, roots, and shoots. The soil was carefully removed from nodules and roots by sieving through a 1-mm sieve. Nodules, roots, and shoots of the plants from each plot were oven-dried at 70˚C for 72 h for the determination of dry weight. The shoot dry matter was powdered in a Cyclotec 1093 sample mill (100 - 120 mesh, Tecator AB, Hoedanaes, Sweden). The N content of the dry matter was analyzed using indophenol [20] after digestion with H_2SO_4-H_2O [31].

For the collection of xylem sap at the R3.5 stage, root bled sap samples from the plants in each plot were taken. To collect the root bled sap, the shoot just under the cotyledon node of each plant was cut with a very sharp cutter according to a previously reported method [32]. Sap samples were kept on ice and frozen at −20˚C for long-term storage. The root bled sap samples were analyzed for amino N [33], NO_3-N [34], and ureide-N [35]. The relative ureide index (RUI) of root bled sap at the R3.5 stage was calculated according to the following formula [32]:

$$\text{RUI}(\%) = 4\times\text{ureide}\big/\left(4\times\text{ureide}+\text{amino acid}+\text{nitrate}\right)\times 100$$

The percentage of N derived from N fixation was calculated from y = 21.3 + 0.67x in which y is relative ureide-N (%) and x is the percentage of N derived from N fixation (%) [36].

At maturity stage, ten continuous hills were harvested by cutting at the cotyledon node of the stem. The plants were harvested from each plot at physiological maturity, leaving the border rows. The yield components parameters of number of pods per plant, number of seeds per pod, and hundred-seed weight were determined. After recording the seed yield, the above-ground dry matters were oven-dried at 70˚C for 72 h for dry weight determination. The dry matters were powdered and N content was analyzed in the same way as for the V6 and R3.5 growth stages.

2.6. Statistical Analysis

Data for the dry weight of nodules, roots and shoots, total N accumulation, RUI, percentage of N derived from the atmosphere, and seed yield were statistically analyzed using STATISTIX 8 (Analytical Software, Tallahassee, FL, USA) and the means were compared by Tukey's HDS test at $P < 0.01$.

3. Results

3.1. Indigenous Rhizobia in the Cultivated Soil

The density of indigenous rhizobia from cultivated soil was estimated by the MPN method. Indigenous rhizobia in this soil nodulated to Yezin-3 (Rj_4) and to Yezin-6 (non-*Rj*) were present at concentrations of 1.16×10^4 and 1.16×10^5 cells (g dry soil)$^{-1}$, respectively.

3.2. Nodulation, Dry Matter Production, and N Accumulation at the V6 and R3.5 Stages

The number of nodules on tap roots per hill was significantly ($P < 0.01$) different between varieties and treatments (**Figure 1**). The Yezin-3 variety had a greater number of nodules on the tap roots than the Yezin-6 soybean variety. A single inoculation of MAS34 resulted in a significantly ($P < 0.01$) higher number of nodules on the tap roots of the Yeizn-3 soybean variety compared to the uninoculated control at the V6 and R3.5 stages. There were no significant differences between P4, MAS34, and the dual inoculation of P4 with MAS34. However, after a single inoculation of MAS34, 32% and 19% of R3.5-stage tap roots of the Yezin-3 soybean variety had higher numbers of nodules than after a single inoculation of P4 and a dual inoculation of P4 with MAS34, respectively. In the Yezin-6 soybean, the dual inoculation of P4 with MAS34 was significantly ($P < 0.01$) higher than the uninoculated control in terms of the number of nodules on the tap roots, but no significant difference was observed between a single inoculation of P4 and MAS34 at the R 3.5 stage. Nevertheless, following the dual inoculation of P4 with MAS34, 35% and 17% of tap roots had a higher number of nodules than after single inoculations of P4 and MAS34 at the R3.5 stage for the Yezin-6 soybean variety. There was no significant difference in the number of nodules on lateral roots per hill (**Figure 1**). However, a single inoculation of MAS34 and the dual inocu-

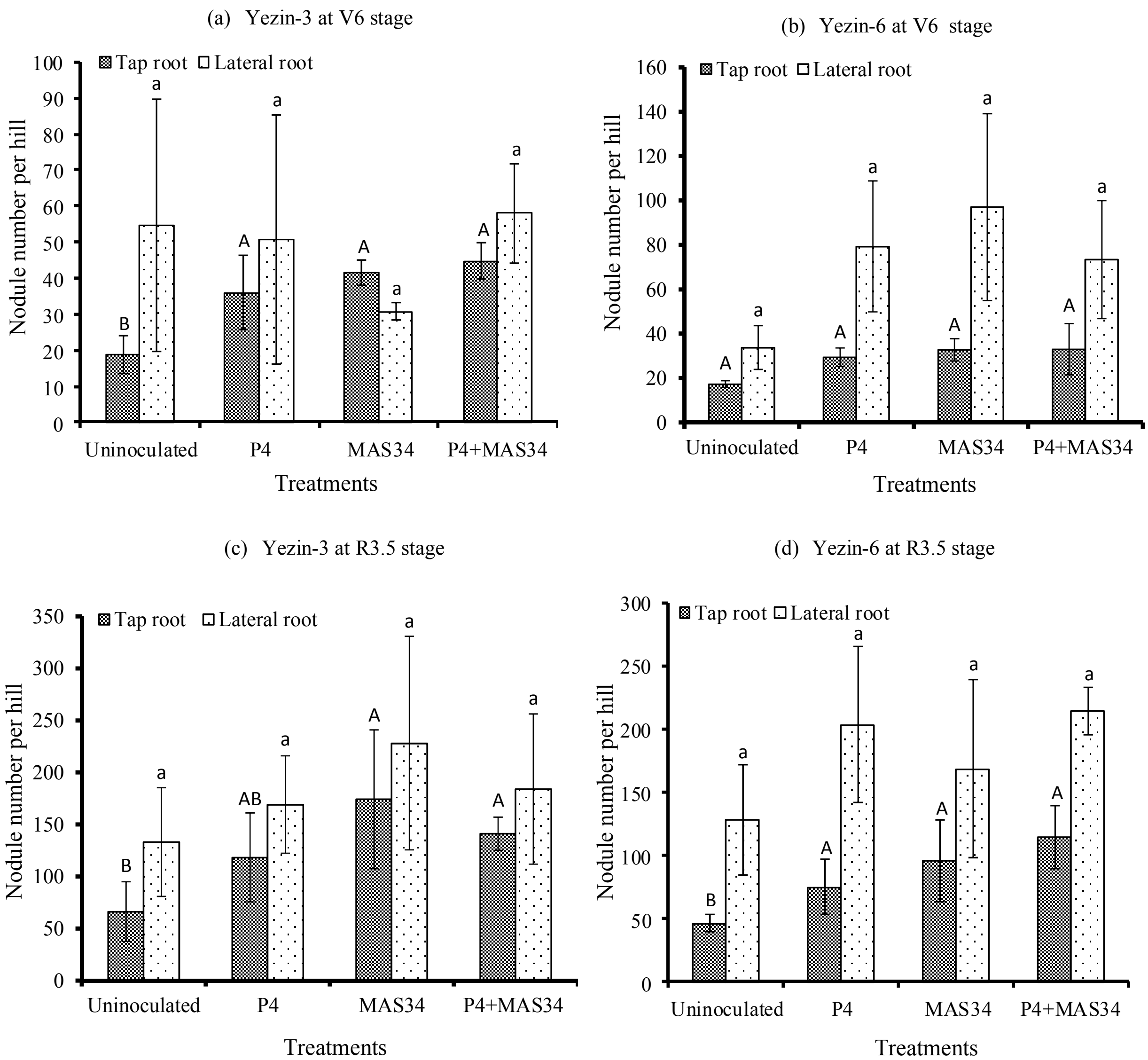

Figure 1. Effects of the co-inoculation of *B. yuanmingense* MAS34 and *S. griseoflavus* P4 on nodule number per hill at different growth stages in the Yezin-3 and Yezin-6 soybean varieties: (a) Yezin-3 at V6, (b) Yezin-6 at V6, (c) Yezin-3 at R3.5, and (d) Yezin-6 at R3.5. Mean values followed by the same letter(s) indicate no significant difference.

lation of P4 with MAS34 resulted in higher numbers of nodules on the lateral roots of the Yeizin-3 and Yezin-6 soybean varieties at both stages. **Table 1** shows the effects of the single and dual inoculations of MAS34 and P4 on nodulation. The efficiency of a single inoculation of MAS34 was high in tap roots at the V6 stage in Yezin-3, but no significant effect was found for nodules on the lateral roots for all treatments at both stages in the Yezin-3 and Yezin-6 varieties.

The dry weight of nodules was significantly ($P < 0.01$) affected by the soybean variety and also the inoculation treatment at the V6 stage (**Table 2**). In contrast, it was not significantly affected by the interaction of the soybean variety and treatment at the V6 stage. All inoculated treatments had a significantly ($P < 0.01$) higher nodule dry weight in both soybean varieties than the uninoculated control. Yezin-3 had a significantly ($P < 0.01$) higher nodule dry weight (0.41 g·hill^{-1}) when inoculated with the single inoculation of *B. yuanmingense* MAS34 than did the Yezin-6 variety (0.22 g·hill^{-1}) that received a dual inoculation of *S. griseoflavus* P4 with MAS34.

The dry weight of nodules was significantly ($P < 0.01$) improved by the inoculated treatment alone at the R3.5 growth stage (**Table 3**). In contrast, it was not significantly affected by the variety alone or by the interaction of soybean variety and inoculation treatment at the R3.5 stage. The nodule dry weights following single and dual inoculations of P4 and MAS34 were significantly ($P <$

Table 1. Effects of co-inoculation of *B. yuanmingense* MAS34 and *S. griseoflavus* P4 on weight per nodule in the Yezin-3 and Yezin-6 soybean varieties at the V6 and R3.5 stages.

Treatment	Weight per nodule (mg)			
	V6 stage		R3.5 stage	
	Tap root	Lateral root	Tap root	Lateral root
Yezin-3				
Uninoculated	6.27 abc	1.22 a	7.90 a	2.61 a
P4	7.10 ab	1.91 a	7.70 ab	4.14 a
MAS34	8.37 a	1.81 a	5.81 ab	3.60 a
P4 + MAS34	6.74 ab	1.78 a	6.98 ab	3.44 a
Yezin-6				
Uninoculated	2.65 c	0.79 a	6.16 ab	2.16 a
P4	3.70 bc	0.97 a	6.43 ab	2.43 a
MAS34	4.12 bc	0.88 a	4.94 ab	2.37 a
P4+MAS34	4.28 bc	0.93 a	4.58 b	2.82 a

Means within a column followed by the same letter(s) indicate no significant difference.

Table 2. Effects of co-inoculation of *B. yuanmingense* MAS34 and *S. griseoflavus* P4 on plant dry weight and total N accumulation in the Yezin-3 and Yezin-6 soybean varieties at the V6 stage.

Treatment	Plant dry weight ($g \cdot hill^{-1}$)			Total N accumulation ($mg\ N \cdot hill^{-1}$)
	Nodule	Root	Shoot	
Varieties				
Yezin-3	0.32 a	1.24 a	5.62 a	172 a
Yezin-6	0.18 b	1.10 a	4.00 a	119 a
Treatment of Yezin-3				
Uninoculated	0.17 b	0.87 b	3.51 b	108 b
P4	0.33 a	1.31 ab	6.04 ab	186 ab
MAS34	0.41 a	1.38 a	6.01 ab	183 ab
P4 + MAS34	0.40 a	1.41 a	6.96 a	211 a
Treatment of Yezin-6				
Uninoculated	0.07 b	0.63 b	2.00 b	56.9 b
P4	0.19 a	1.08 a	3.83 a	116 a
MAS34	0.22 a	1.25 a	4.66 a	141 a
P4 + MAS34	0.20 a	1.43 a	5.23 a	160 a
Tukey HSD test				
Variety	**	NS	NS	NS
Treatment	**	**	**	**
Variety × Treatment	NS	NS	NS	NS
CV%	10.9	12.6	14.7	16.3

NS, **: nonsignificant or significant at $P < 0.01$ respectively. Mean values followed by the same letter(s) indicate no significant difference.

0.01) different in Yezin-3. However, in Yezin-6, the single P4 inoculation did not significantly affect nodulation at the R3.5 stage. The single inoculation of MAS34 produced a significantly ($P < 0.01$) higher nodule dry weight in Yezin-3 but the dual inoculation of P4 + MAS34 did not produce the same result in Yezin-6.

The dry matter production at the V6 stage was significantly ($P < 0.01$) different between the different inoculation treatments (**Table 2**). It was not significantly affected by the soybean variety. The Yezin-3 soybean variety produced a significantly higher amount of dry matter (1.41 g·hill^{-1} in roots; 6.96 g·hill^{-1} in shoots) following the dual inoculation of P4 and MAS34. The same response was found in Yezin-6 (1.43 g·hill^{-1} in roots; 5.23 g·hill^{-1} in shoots) with the same dual inoculation of P4 and MAS34. The single inoculation of P4 produced a significant response compared to the uninoculated control in Yezin-6, but there was no significant effect on dry matter production in Yezin-3 at the V6 stage. The dual inoculation of P4 and MAS34 resulted in the highest dry matter production regardless of soybean variety. However, the interaction between variety and treatment was found to be not significant in relation to dry matter production.

The dry matter production at the R3.5 growth stage was significantly ($P < 0.01$) increased following the inoculation treatments (**Table 3**). However, there was no significant difference between the soybean variety and the interaction of variety and treatment. The Yezin-3 soybean variety produced a significantly higher amount of dry matter following the single inoculation of MAS34. However, the dual inoculation of P4 with MAS34 significantly increased dry matter production in Yezin-6. The single inoculation of P4 did not significantly affect dry matter production in Yezin-3 and Yezin-6 compared with the uninoculated control at the R3.5 stage.

Table 3. Effects of co-inoculation of *B. yuanmingense* MAS34 and *S. griseoflavus* P4 on plant dry weight and total N accumulation in the Yezin-3 and Yezin-6 soybean varieties at the R3.5 stage.

Treatment	Plant dry weight (g·hill^{-1})			Total N accumulation (mg N·hill^{-1})
	Nodule	Root	Shoot	
Varieties				
Yezin-3	1.42 a	14.3 a	60.5 a	220 a
Yezin-6	1.36 a	13.5 a	56.0 a	206 a
Treatment of Yezin-3				
Uninoculated	0.84 b	13.0 b	50.1 b	196 a
P4	1.51 a	14.5 ab	61.5 ab	211 a
MAS34	1.77 a	15.0 a	65.8 a	238 a
P4 + MAS34	1.52 a	14.9 a	64.3 a	236 a
Treatment of Yezin-6				
Uninoculated	0.56 b	11.8 b	48.3 b	189 a
P4	0.94 ab	13.5 ab	53.8 ab	198 a
MAS34	0.83 ab	14.2 a	58.7 ab	211 a
P4+MAS34	1.14 a	14.5 a	63.0 a	224 a
Tukey HSD test				
Variety	NS	NS	NS	NS
Treatment	**	**	**	*
Variety × Treatment	NS	NS	NS	NS
CV%	10.9	4.29	7.76	9.07

NS, *, **: nonsignificant or significant at $P < 0.05$ or $P < 0.01$ respectively. Mean values followed by the same letter(s) indicate no significant difference.

The effects of the co-inoculation of Myanmar *B. yuanmingense* MAS34 and *S. griseoflavus* P4 in enhancing the total N accumulation at the V6 and R3.5 stages of the two soybean varieties are presented in **Tables 2** and **3**. The total N accumulation by the soybeans at the V6 stage was significantly ($P < 0.01$) different between the inoculated treatments. The effect of variety and the interaction of variety with treatment had no significant influence on the total N accumulation by the soybeans at the V6 stage (**Table 2**). The dual inoculation of P4 with MAS34 in Yezin-3 significantly ($P < 0.01$) influenced the total N accumulation compared to the other treatments and the uninoculated control. All inoculated treatments were significantly ($P < 0.01$) better than the uninoculated control at the V6 stage in Yezin-6.

The total N accumulation at the R3.5 stage was also observed to be significantly ($P < 0.01$) affected by the inoculation treatments. There was no effect of the variety or of the interaction of variety with treatment on the total N accumulation by soybeans at the R3.5 stage (**Table 3**). There was an insignificant effect on the total N accumulation in Yezin-3 and Yezin-6 compared with the unionculated control at the R3.5 stage (**Table 3**). A higher total N accumulation was observed following the dual inoculation of P4 with MAS34 in Yezin-6 and the single inoculation of MAS34 in Yezin-3.

3.3. N_2 Fixation at R3.5 Stage

The RUI of root bled sap of soybean at the R3.5 growth stage [37] was used to indicate the extent of N_2 fixation resulting from the inoculation treatment and the percentage of plant N derived from the atmosphere within the soybean growing season. The RUI and the percentage of plant N derived from the atmosphere resulting from the co-inoculation of *B. yuanmingense* MAS34 and *S. griseoflavus* P4 in Yezin-3 and Yezin-6 at the R3.5 stage are presented in **Table 4**.

Table 4. Effects of co-inoculation of *B. yuanmingense* MAS34 and *S. griseoflavus* P4 on N_2 fixation in the Yezin-3 and Yezin-6 soybean varieties at the R3.5 stage.

Treatment	Relative ureide index (%)	Percentage of plant N derived from atmosphere N (%)
Varieties		
Yezin-3	67.9 a	69.5 b
Yezin-6	79.2 a	86.5 a
Treatment of Yezin-3		
Uninoculated	53.4 b	47.8 b
P4	68.9 ab	71.1 ab
MAS34	77.8 a	84.4 a
P4 + MAS34	71.4 ab	74.8 ab
Treatment of Yezin-6		
Uninoculated	74.3 b	79.1 b
P4	74.5 ab	79.4 ab
MAS34	80.3 ab	88.1 ab
P4+MAS34	87.8 a	99.3 a
Tukey HSD test		
Variety	NS	*
Treatment	**	**
Variety × Treatment	NS	NS
CV%	7.98	11.7

NS, *, **: nonsignificant or significant at $P < 0.05$ or $P < 0.01$ respectively. Mean values followed by the same letter(s) indicate no significant difference.

The RUI and percentage of plant N derived from the atmosphere were significantly ($P < 0.01$) different between the inoculation treatments. They were not significantly ($P < 0.01$) affected by the variety and the interaction of variety and inoculation treatment. In Yezin-3, a single inoculation of MAS34 (77.8%) significantly ($P < 0.01$) improved the RUI at the R.5 stage compared to the uninoculated control (53.2%). The percentage of plant N derived from the atmosphere from the single inoculation of MAS34 was about 84.4%. There was no significant difference between single and dual inoculations of P4 on the RUI of root bled sap from Yezin-3. The RUI values of the treatments were within the range of 68.9% - 71.4%, and plant N derived from N_2 fixation ranged between 71.1% and 74.8%. The single inoculation of P4 did not improve the N_2 fixation of soybean, but this treatment did increase N_2 fixation by about 15% over that of the uninoculated control.

Yezin-6 from the uninoculated control had an RUI value of about 74.3% for root bled sap at the R3.5 stage. The single inoculations of P4 and MAS34 had no significant effect on the RUI, and they were statistically equivalent to the uninoculated control. The dual inoculation of P4 with MAS34 (87.8%) resulted in a significant ($P < 0.01$) increase in the RUI compared to the other inoculated treatments and the uninoculated control (74.1%) in Yezin-6. The percentage of plant N derived from the atmosphere in the Yezin-6 soybean variety following the dual inoculation of P4 with MAS34 was about 99.3% and significantly different ($P < 0.01$) from the other treatments. The percentages of plant N derived from the atmosphere from P4 (79.4%) and MAS34 (88.1%) were higher than that of the uninoculated control (79.1%).

3.4. Above-Ground Dry Biomass at the Maturity Stage

The treatment and variety-treatment interaction had a significant ($P < 0.05$) effect on the above-ground dry biomass yield. The single inoculation of MAS34 produced a significantly larger dry biomass yield (80.4 $g \cdot hill^{-1}$) in Yezin-3 compared to P4 (62.3 $g \cdot hill^{-1}$) and the uninoculated control (41.4 $g \cdot hill^{-1}$). In Yezin-6, the dual inoculation of P4 + MAS 34 significantly increased the dry biomass (84.7 $g \cdot hill^{-1}$) compared to P4 (66.3 $g \cdot hill^{-1}$) and the uninoculated control (44.7 $g \cdot hill^{-1}$). In this study, the variety alone did not produce a significant response in the above-ground dry biomass yield (**Figure 2**).

3.5. Total N Accumulation at Maturity

The total nitrogen uptake by soybean plants at maturity was significantly ($P < 0.01$) different between the inoculated treatments and the variety-treatment interaction. The inoculation of *B. yuanmingense* MAS34 resulted in the maximum N accumulation (139 $kg \cdot N \cdot ha^{-1}$) at maturity in Yezin-3, which was significantly higher than with the inoculation of P4 and in the uninoculated control (**Table 4**). As in Yezin-6, the dual inoculation of P4 with MAS 34 resulted in a higher N accumulation (148 $kg \cdot N \cdot ha^{-1}$) at maturity than in the uninoculated control (**Figure 3**).

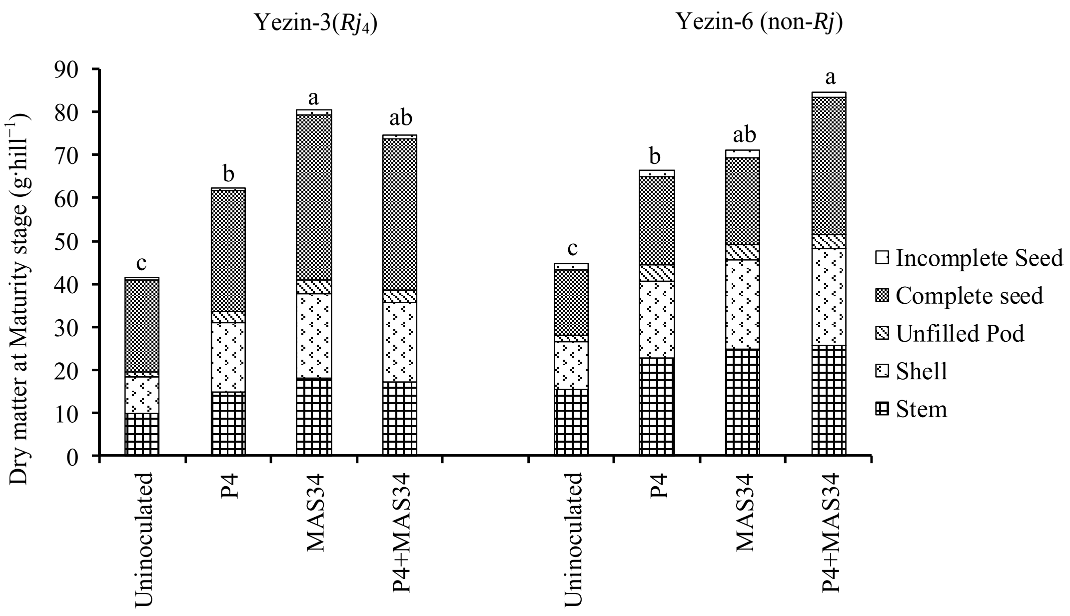

Figure 2. Effects of the co-inoculation of *B. yuanmingense* MAS34 and *S. griseoflavus* P4 on dry matter at the maturity stage in the Yezin-3 and Yezin-6 soybean varieties; the four treatments of uninoculated, P4, MAS34, and P4 + MAS34 are shown on the horizontal axis. Mean values followed by the same letter(s) indicate no significant difference.

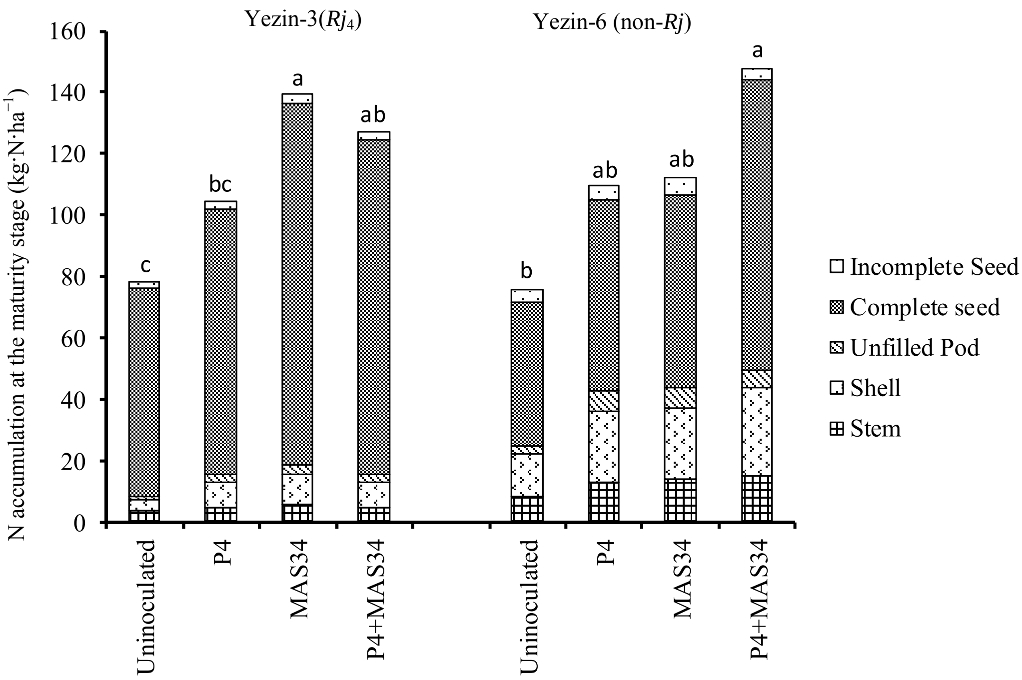

Figure 3. Effects of the co-inoculation of *B. yuanmingense* MAS34 and *S. griseoflavus* P4 on N accumulation at the maturity stage in the Yezin-3 and Yezin-6 soybean varieties; the four treatments of uninoculated, P4, MAS34, and P4 + MAS34 are shown on the The English in this document has been checked by at least two professional editors, both native speakers of English. For a certificate, please see: http://www.textcheck.com/certificate/rqZzin.

3.6. Yield and Yield-Related Parameters

The number of pods per plant was significantly ($P < 0.01$) different among the inoculation treatments in both Yezin-3 and Yezin-6. The single inoculation of MAS34 resulted in a significantly higher number of pods per plant than the single inoculation of P4 and the uninoculated control in Yezin-3, but it was not statistically different from the P4 + MAS 34 treatment. In Yezin-6, the dual inoculation of P4 + MAS34 was significantly different to the unioncultated control. The effect of the soybean variety alone was not significant in terms of the number of pods per plant, and the interaction of variety and inoculation treatment also had a non-significant influence on the number of pods per plant (**Table 5**).

Although the interaction of variety and treatment did not significantly influence the number of seeds per pod, the effect of inoculation treatment and variety alone was significant ($P < 0.01$). The single inoculation of MAS34 and the dual inoculation of P4 + MAS34 resulted in a significantly higher number of seeds per pod than in the uninoculated control in Yezin-3. In Yezin-6, there was no significant difference between each treatment (**Table 5**).

Hundred-seed weight was found to be significantly ($P < 0.01$) affected by the effect of inoculation treatment as well as the interaction between soybean variety and treatment. The single inoculation of MAS34 in Yezin-3 and the dual inoculation of P4 + MAS34 in Yezin-6 responded significantly. However, the main effect of soybean varieties alone was non-significant in relation to the hundred-seed weight (**Table 5**).

Seed yield varied significantly ($P < 0.01$) due to the effects of both soybean variety and inoculation treatment (**Table 5**). The higher seed yield in Yezin-3 may be attributed to the larger number of pods per plant recorded for this variety. The analysis of variance indicated that variety and treatment had an interaction effect on the seed yield. The increase in seed yield was significant ($P < 0.01$) following the single inoculation of MAS34 (3.45 ton·ha^{-1}) in Yezin-3 compared to the uninoculated control. Significant ($P < 0.01$) increases in seed yield were recorded following the dual inoculation of P4 + MAS34 (2.90 ton·ha^{-1}) in Yezin-6.

4. Discussion

A successful *Rhizobium*-legume symbiosis largely depends on the presence of a specific and compatible strain in the soil for a particular legume. Several studies have reported a significant increase in soybean growth parameters and yield due to the inoculation of bradyrhizobial isolates [38-40].

Many studies noted that soybean plant nodulation was inhibited when plants were inoculated with high-density cell inoculum [12,41-43]. Furthermore, significantly higher nodulation, fixed nitrogen, and seed yields were

Table 5. Effects of co-inoculation of *B. yuanmingense* MAS34 and *S. griseoflavus* P4 on seed yield and yield components at the maturity stage in theYezin-3 and Yezin-6 soybean varieties.

Treatment	Pods per hill	Seeds per pod	Hundred-seed weight (g)	Yield (ton·ha $^{-1)}$)
Varieties				
Yezin-3	151 a	1.54 a	14.0 a	2.76 a
Yezin-6	165 a	1.11 b	13.0 a	2.02 b
Treatment of Yezin-3				
Uninoculated	131 c	1.39 b	12.5 b	1.93 c
P4	139 bc	1.54 ab	14.9 a	2.52 bc
MAS34	177 a	1.65 a	14.9 a	3.45 a
P4 + MAS34	158 ab	1.57 a	14.5 a	3.14 ab
Treatment of Yezin-6				
Uninoculated	145 b	1.01 a	11.7 b	1.42 b
P4	151 b	1.13 a	13.2 ab	1.89 ab
MAS34	170 ab	1.07 a	12.4 ab	1.86 ab
P4 + MAS34	191 a	1.24 a	14.5 a	2.90 a
Tukey HSD test				
Variety	NS	**	NS	**
Treatment	**	**	**	**
Variety × Treatment	NS	NS	*	*
CV%	6.74	6.94	4.81	12.9

NS, *, **: nonsignificant or significant at $P < 0.05$ or $P < 0.01$ respectively. Mean values followed by the same letter(s) indicate no significant difference; 8.33 hills per square meter.

observed in plants inoculated by low concentrations of USDA110 under field conditions [12]. In our study, plant biomass, nitrogen uptake, and seed yield in the Yezin-3 (Rj_4) and Yezin-6 (non-*Rj*) soybean varieties were clearly enhanced at Kyushu University Farm when they were inoculated with a low density of (10^5 cell·seed^{-1}) inoculums of *B. yuanmingense* MAS34 and *S. griseoflavus* P4.

In this field experiment, soybean plants were grown in cultivated soil in an open field using ordinary water for irrigation throughout the experimental period. The plants from the uninoculated control and the *S. griseoflavus* P4 treatment were able to form root nodules, indicating that the indigenous rhizobia in the soil is nodulated to soybean varieties in the open field. This is in line with previous findings [44] that the response of legumes to inoculation depends largely on the number of rhizobia already established in the soil, the availability of soil N, and the demand for N by the crop.

The first root nodules formed on the basal part of the primary roots becoming visible about 10 days after planting. They started to fix nitrogen (N_2) at about 15 - 20 days after planting when the diameter reached approximately 2 mm [45]. In the later stages, the nodules formed at the basal part of primary roots degrade, and a large number of new nodules formed on the lateral roots near the soil surface. These nodules play an important role in supplying N during the pod filling stage. In our study, a number of nodules formed on the tap roots and lateral roots of soybeans in accordance with the above description and resulted in an improved seed yield.

Souleimanov *et al*. [46] found that the Nod factor of *B. japonicum* had an effect on root growth that resulted in a 34% - 44% longer root in soybean. Significant effects on root biomass improvement in pea and lentil following seed treatment with strains of *Rhizobium legumanosarum* bv. *viceae* in field experiments have also been reported [47]. Studies have also shown results similar to our finding that a single inoculation of *B. yuanmingense* MAS34 increases root dry weight in Yezin-3 and Yezin-6 soybeans at the V6 and R3.5 stages.

In our study, a single inoculation of *B. yuanmingense* MAS34 resulted in a significant improvement in N_2 fixation in Yezin-3 but only a positive trend in Yezin-6. This supports the conclusions of Wani *et al*. [48] that within grain legume species, genotypic variability affects the nodule number or nitrogenase activity. Comparative assessment of the field trial results suggested that *B. yuanmingense* MAS34 consistently resulted in significant improvements in nodulation, dry matter production, N_2 fixation, and seed yield in Yezin-3. Increased nodulation and subsequent nitrogen fixation resulted in increased plant growth and grain yield. Similarly, Egamberdiyeva *et al*. [49] noticed a positive effect of inoculation with *B. japonicum* S2492 on growth, nodule number, and yield of soybean under field conditions.

Milic *et al*. [50] also reported variability in the dry matter mass and nitrogen content in the nodules of soybean varieties following the use of different bradyrhizobial strains. Such variations may be attributed to differences in the genomic constitution of the host or bacteria or both, which control symbiosis, or there might be more than one affinity group within the legume rhizobia. According to Okereke *et al*. [7] the inoculation response of *Bradyrhizobium* in different soybean cultivars is cultivar- and site-specific. We also observed variations between the two tested soybean varieties in terms of their response to bradyrhizobial strain.

In both soybean varieties, *S. griseoflavus* P4 had a significant effect only on the improvement of plant dry weight at the V6 stage and dry matter production at maturity was significantly better than in the uninoculated control. Soe *et al*. [15] stated that *S. griseoflavus* P4 alone could improve the shoot dry weight of Myanmar soybean varieties. In the present study, the co-inoculation of MAS34 with P4 significantly improved nodule dry weight, plant biomass, N_2 fixation, and seed yield in Yezin-6 but not in Yezin-3. This supports the results of our previous study [51], in which the dual inoculation of endophytic actinomycetes (*Streptomeces* sp. strain, P4) with bradyrhizobial strains increased the nodulation and nitrogen fixation in some soybean varieties but not in other varieties.

Rj genes play a role in controlling the plant's compatibility with specific rhizobial strains and also the preference for indigenous soybean-nodulating rhizobia [26,27]. In addition, non-*Rj* is compatible with all bradyrhizobial strains but Rj_4 has unique features that restrict nodulation with specific strains of *Bradyrhizobium* [28]. In this study, Yezin-3 (Rj_4) responded significantly to the single inoculation of *B. yuanmingense* MAS34. The MAS34 was isolated from a Rj_4-genotype host [15]. These findings support the results of previous studies [26-28]. The *Rj*-genotype of soybeans appears to be compatible with the preference of bradyrhizobial strains for soybean cultivation. Inoculation with effective strains of bradyrhizobia may promote soybean growth and seed yield. We found that the symbiotic interaction of the P4 with *B. yuanmingense* MAS34 significantly improved nodule dry weight, nitrogen fixation, and seed yield in Yezin-6. This synergistic efficacy of *S. griseoflavus* P4 with *B. yuanmingense* MAS34 was found in Yezin-6 (non-*Rj*). This also supports previous findings showing that the dual inoculation of bradyrhizobial strains and *S. griseoflavus* P4 increased nodulation and nitrogen fixation in different soybean varieties [51,52].

Synergistic effects of the co-inoculation of *S. griseoflavus* P4 with *B. yuanmingense* MAS34 have been found in Yezin-6 grown at Kyushu University Farm, Japan. This positive interaction was observed under standard environmental conditions, using the correct varieties, and proper nodulated bacteria in conjunction with *S. griseoflavus* P4. Our experimental results confirm the findings of Akarapisan *et al*. [52] and Soe *et al*. [51] that *S. griseoflavus* P4 is an effective endophytic actinomycete, which can be used in combination with selective root nodule bacterial strains for the production of economically important leguminous crops. Further experimental investigations of the synergistic effectiveness of selected *B. yuanmingense* MAS34 and *S. griseoflavus* P4 with different soybean cultivars are needed to determine the optimal Myanmar soybean growing environment where this MAS34 was isolated. We envisage that the Myanmar *Bradyrhizobium* strain and *S. griseoflavus* P4 will be useful as biofertilizers for soybean production in the future.

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