American Journal of Plant Sciences, 2015, 6, 2799-2810
Published Online November 2015 in SciRes. http://www.scirp.org/journal/ajps
http://dx.doi.org/10.4236/ajps.2015.618276

Determining Nodulation Regulatory (*Rj*) Genes of Myanmar Soybean Cultivars and Their Symbiotic Effectiveness with *Bradyrhizobium japonicum* USDA110

Aung Zaw Htwe[1*], Yuichi Saeki[2], Kyi Moe[3], Takeo Yamakawa[4]

[1]Laboratory of Plant Nutrition, Graduate School of Bioresource and Bioenvironmental Sciences, Faculty of Agriculture, Kyushu University, Fukuoka, Japan
[2]Department of Biochemistry and Applied Biosciences, Faculty of Agriculture, Miyazaki University, Miyazaki, Japan
[3]Department of Agronomy, Yezin Agricultural University, Naypyitaw, Myanmar
[4]Laboratory of Plant Nutrition, Division of Molecular Biosciences, Department of Bioresource and Bioenvironmental Sciences, Faculty of Agriculture, Kyushu University, Fukuoka, Japan
Email: *aungzawhtwe333@gmail.com

Received 2 October 2015; accepted 6 November 2015; published 11 November 2015

Abstract

Soybean (*Glycine max* L.) plays an essential role in human nutrition as a protein source, and in plant nutrition as a N source. The rate of N fixation varies depending on the cultivars and compatibility between the inoculated *Rhizobium* strain and the host cultivar. Characterizing the nodulation regulatory (*Rj*) genes is necessary to determine the compatibility of cultivars and *Rhizobium* strains. *Rj* genes were previously identified based on inoculation tests and PCR analyses. The six cultivars Yezin-3, Yezin-7, Yezin-11, Shan Seine (Local), Madaya (Local), and Hinthada (Local) were identified as harboring the Rj_4 gene. Two cultivars, Yezin-6 and Yezin-8, were classified as *non-Rj*-gene harboring. Two other cultivars, Yezin-9 and Yezin-10, were identified as Rj_3- and Rj_2Rj_3-gene harboring, respectively. Ours is the first report on Rj_3- and Rj_2Rj_3-gene harboring cultivars in Myanmar. We evaluated Myanmar soybean cultivars for symbiotic effectiveness, relying on the standard strain *Bradyrhizobium japonicum* USDA110. In our first experiment, the soybean cultivar Yezin-11 (Rj_4) showed the highest N fixing potential. Based on their potential for fixing N and nodulation, the top six soybean cultivars were Yezin-11 (Rj_4), Yezin-9 (Rj_3), Yezin-6 (*non-Rj*), Yezin-8 (*non-Rj*), Yezin-3 (Rj_4) and Yezin-10 (Rj_2Rj_3). These cultivars were selected for a second experiment, which revealed that the N fixation, nodulation, and plant growth of Yezin-11 (Rj_4)

*Corresponding author.

How to cite this paper: Htwe, A.Z., Saeki, Y., Moe, K. and Yamakawa, T. (2015) Paper Determining Nodulation Regulatory (*Rj*) Genes of Myanmar Soybean Cultivars and Their Symbiotic Effectiveness with *Bradyrhizobium japonicum* USDA110. *American Journal of Plant Sciences*, **6**, 2799-2810. http://dx.doi.org/10.4236/ajps.2015.618276

were superior to the other cultivars. We conclude that Yezin-11 (Rj_4) is the most efficient cultivar for nodulation and N fixation when inoculated with *B. japonicum* USDA110.

Keywords

***B. japonicum* USDA110, Inoculation Test, PCR Analysis, Nodulation Regulatory Genes (*Rj* Gene), Symbiotic Effectiveness**

1. Introduction

Myanmar is an agricultural country, as agriculture is the backbone of its economy. Legumes are the second largest crops in Myanmar, following rice (*Oryza sativa* L.), in terms of cultivated hectares. Due to the relatively low cost of cultivation and increasing demand for domestic consumption and export, the total cultivated area of pulses has increased from 0.73 million hectares in 1988-89 to 4.4 million hectares in 2011-12 [1]. Soybean (*Glycine max* L.) is an important cash crop and the second largest cultivated crop after rice in Myanmar [2]. Soybean is also one of the most efficient leguminous crops in terms of fixing N [3] [4].

Nodule formation by a cultivar is often dependent on a specific *Rhizobium* strain [5], which may be attributed to nodulation regulatory genes called *Rj* genes. Different soybean cultivars possess different nodulation *Rj* genes. In soybean, the alleles *Rj*(s) and *rj*(s) are dependent on their compatibility with *Bradyrhizobium* and *Ensifer*/*Sinorhizobium* species [6]. Some nodulation *Rj* genes are found in nature, while others resulted from artificially induced mutations [6]. Williams and Lynch [7] found a non-nodulating soybean line, the rj_1 genotype, which resulted from a cross between the cultivars Lincoln and Richard. The *Rj* genes Rj_2, Rj_3 and Rj_4 inhibit the formation of functional nodules by certain *Bradyrhizobium* strains [8]-[11]. The regulatory gene *Rfg*1 restricts nodulation by the fast-growing strain *Sinorhizobium fredii* USDA257 [12].

Ishizuka *et al.* [13] [14] tested the compatibility and preference of the *Rj*-genotype with specific *Bradyrhizobium* strains. *Bradyrhizobium* strains are classified into nodulation Types A, B, and C based on their compatibility with *Rj* cultivars. Type A strains are capable of forming nodules on all *Rj* genotype cultivars. Type B strains cannot form nodules on the Rj_2Rj_3-gene harboring cultivars. Type C strains are inhibited from nodule formation by Rj_4 genotype cultivars. When different *Rj*-gene harboring cultivars are planted in the same field, non-*Rj*, Rj_4 and Rj_2Rj_3 cultivars selectively form nodules with the Types A, B and C strains, respectively. Many scientists reported that the indigenous *Bradyrhizobium* strains in the soil exhibited preferences for nodulation on compatible *Rj* genotypes [15]-[18]. Recently, Soe *et al.* [19] identified Myanmar soybean cultivars with *non-Rj* and Rj_4 genotypes. Soe *et al.* [20] [21] pointed out that the cultivars Yezin-6, harboring the *non-Rj* gene, and Yezin-3, harboring the Rj_4 gene, had enhanced nodulation and N fixation when inoculated with *B. japonicum* USDA110 and indigenous strains. Yamakawa *et al.* [22] [23] stated that $Rj_2Rj_3Rj_4$ conferred improved nodulation when inoculated with *B. japonicum* USDA110. *Bradyrhizobium japonicum* USDA110, which is a Type A strain, could form functional nodules in all dominant *Rj* genotypes. Therefore, *B. japonicum* USDA110 is used in many countries as an inoculant to increase soybean yield.

The *Rj* genes are identified by an inoculation test, which uses strains that restrict nodulation on specific *Rj* genotype soybean cultivars. As an accelerated method for molecularly identifying *Rj* genes, Yang *et al.* [24], Tang *et al.* [25] and Hayashi *et al.* [26] used cloning to identify the genes Rj_2, *Rfg*1 and Rj_4. Yang *et al.* [24] classified the *Rj*2 and *Rfg*1 genes that encoded a member of the Toll-interleukin receptor/nucleotide-binding site/leucine-rich repeat (TIRNBS- LRR) class of plant resistance (R) proteins involved in host resistance to microbial pathogens through an effector-triggered immune (ETI) response. Recently, Hayashi *et al.* [26] described the molecular identification of the Rj_4 gene based on map-based cloning of several mapping populations. They identified the Rj_4 genes that encoded a thaumatin-like protein (TLP) belonging to the pathogenesis-related (PR) protein family 5, which was involved in inhibition of nodulation with specific *Rhizobia* strains. Cloning of the Rj_3 gene has not been reported, so its identification is only based on inoculation test results.

In Myanmar, many researchers have been focusing on selecting strains to increase soybean N fixation. Recently, the Department of Agricultural Research (DAR) has developed improved soybean varieties, such as Yezin-9, Yezin-10, and Yezin-11. However, *Rj* genes have not been identified in some of the released cultivars. To

recommend the most efficient N-fixing cultivars, it is necessary to evaluate symbiotic effectiveness with inoculated strains and identify the nodulation *Rj* genes. In the past, *Rj* genes were identified based on inoculation tests. Therefore, our goal in this study was to identify *Rj* genes of Myanmar soybean cultivars based on inoculation tests and multiplex PCR analysis and to screen the cultivars for N-fixing efficiency by using the standard strain *B. japonicum* USDA 110.

2. Materials and Methods

2.1. Origin of Soybean Varieties

Ten soybean varieties (Shan Seine [local], Hinthada [local], Madaya [local], Yezin-3, Yezin-6, Yezin-7, Yezin-8, Yezin-9, Yezin-10, Yezin-11) were collected from the Food Legume Section, Department of Agricultural Research, Yezin, Myanmar. These varieties were grown in the glasshouse of the Plant Nutrition Laboratory, Kyushu University, Japan from July to November 2013 to obtain genetically pure and viable seeds. The focus was to study the ability of cultivars to adapt to weather in Japan. Shan Seine [local], Hinthada [local], Madaya [local] were widely grown in Shan State, Ayeyawaddy Region, Mandalay and Sagaing Regions, respectively. Yezin cultivars used in this experiment were mainly grown in Yezin, Mandalya Region and Shan State, and recommended for farmers to improve soybean production. Flower color, days to maturity and origin of these varieties are shown in **Table 1**.

2.2. Determination of Nodulation Regulatory Genes by Inoculation Test

The *Rj* genotypes of 10 soybean cultivars, including three reference cultivars D51 (Rj_3), CNS (Rj_2Rj_3) and Hill (Rj_4), were investigated to estimate their compatibility with native bradyrhizobia. These varieties were inoculated with the three bradyrhizobial strains *B. japonicum* Is-1, *B. elkanii* USDA33 and *B. japonicum* Is-34 [13]. The strains Is-1, USDA33 and Is-34 failed to produce nodules on the roots of soybean cultivars harboring the Rj_2Rj_3, Rj_3 and Rj_4 genes, respectively [10] [27].

The seeds were sterilized by soaking them in 2.5% sodium hypochlorite solution for 5 min, rinsing five times with 10 mL of 99.5% ethanol, and washing five times with sterilized half-strength modified Hoagland Nutrient (MHN) solution [28]. Five surface-sterilized seeds were sown in pots filled with 1 L of vermiculite and 0.6 L of N-free MHN solution. The strains mentioned above were cultured in A1E liquid media [29] and incubated on a rotary shaker at 30˚C for 7 days. Inoculant was prepared by diluting 1 mL of liquid bacterial culture with 99 mL of sterilized MHN solution to obtain a bacterial suspension of about 10^7 cells·mL^{-1}. Seeds were inoculated with the bacterial suspension at 5 mL per seed.

Table 1. Origin of Myanmar soybean varieties.

Variety	Flower color	Days to maturity	Origin
Yezin-3	Violet	115	DAR, Yezin, MR
Yezin-6	Violet	115	DAR, Yezin, MR
Yezin-7	Violet	100	DAR, Yezin, MR
Yezin-8	Violet	115	DAR, Yezin, MR
Yezin-9	White	120	DAR, Yezin, MR
Yezin-10	White	120	DAR, Yezin, MR
Yezin-11	Violet	110	DAR, Yezin, MR
Shan Seine	Violet	120	Shan State
Hinthada	Violet	120	Hinthada, AR
Madaya	Violet	130	Madaya, MR

DAR: Department of Agricultural Research; AR: Ayeyawaddy Region; MR: Mandalay Region.

The plants were cultivated in an environmentally-controlled room (25˚C and 75% Relative Humidity) under natural light for 4 weeks. Control pots were used to check for contamination by non-relevant strains and inoculated strains used in this experiment. Watering was done weekly with autoclaved deionized water. After 1 month, the formation of effective nodules was checked to identify nodulation types of all isolates being tested. This experiment was conducted three times, from January to June 2015.

2.3. Determination of Nodulation Regulatory Genes by PCR Analysis

Multiplex PCR analysis was used to identify *Rj* genes and confirm the Rj_2 and Rj_4 alleles, but it could not detect the Rj_3 allele. For DNA extraction, the plants were cultivated in a growth chamber (28˚C for 16 hours for the light condition and 23˚C for 8 hours for the dark condition). Genomic DNA for PCR templates was extracted from the leaves of seedlings using Takara Bio, following the manufacturer's instructions. Primers were designated from sequence information in reports identifying the Rj_2 and Rj_4 genes [24]-[26]. The primers are described in **Table 2**. The PCR reaction consisted of a pre-run at 94˚C for 5 min, denaturation at 94˚C for 30 s, annealing at 65˚C for 30 s, and extension at 72˚C for 30 s for the first 10 cycles, with a decrease in annealing temperature of 1˚C per cycle. The remaining 20 cycles were repeated at the same temperatures for denaturing and annealing, annealing at 55˚C, and extension at 72˚C for 30 s, followed by the final extension at 72˚C for 10 min and preservation at 4˚C. The reaction producer of PCR analysis of *Rj* genes was innovated by Dr. Yuichi Saeki (Professor, Department of Biochemistry and Applied Biosciences, Miyazaki University). Photos of PCR products were taken after agarose gel electrophoresis (3% agarose gel in 1x TAE buffer) to check the band placement and identify the *Rj* genes.

2.4. Evaluation of Symbiotic Effectiveness of Myanmar Soybean Cultivars

The seeds were surface-sterilized as described above. Six surface-sterilized seeds were sown in pots filled with 1 L of vermiculite and 0.6 L of N-free MHN solution. *Bradyrhizobium japonicum* USDA110 was cultured in A1E liquid media and incubated on a rotary shaker at 30˚C for 7 days. Inoculant was prepared as described above. Seeds were inoculated with the bacterial suspension at 5 mL per seed. The cultivation conditions were the same as described above. Three plants were chosen from each pot for data collection in the first experiment. Six plants were taken from three different pots for the second experiment.

For the acetylene reduction assay (ARA), the soybean plants with intact nodules were placed in 100-mL conical flasks, sealed with a serum stopper and injected with 12 mL of acetylene (C_2H_2) gas to replace air with acetylene.

Table 2. Primers sets and amplification of Multiplex PCR analysis.

Primer sets	Primer sequences	Annealing temperature (˚C)	Amplification site (bp)
Rj_2 specific primers			
K452E-F	(5'-GCTTCAATAGATATGACTTGACAG-3')	58.5	161
R490I-R	(5'-AATCAAGTCATGCATTGTAACTA-3')	57.8	
rj_2 specific primers			
K452-F	(5'-GCTTCAATAGATATGACTTGACAA-3')	58.9	161
R490-R	(5'-ATCAAGTCATGCATTGTAACTC-3')	58.2	
Rj_4 specific primers			
T107A-F	(5'-TTGGAGGAAACGCCG-3')	62.2	324
202-203AY-R	(5'-AATCATGAGAAGAACAAGTATAAGC-3')	58.5	
rj_4 specific primers			
T107-F	(5'-TTGGAGGAAACGCCA-3')	59.9	318
202-203del-R	(5'-AATCATGAGAAGAACAAGTATGGA-3')	60.4	

The nitrogenase activity, in terms of ethylene (C_2H_4) concentration of the plants, was measured using a flame ionization gas chromatograph (GC-14A, Shimadzu, Kyoto, Japan) at 5 and 65 min after injecting with C_2H_2 gas as described by Soe *et al.* [20]. After completing the assay, nodules were counted by removing them from the roots. Shoots, roots, and nodules were collected separately and oven dried at 70˚C for 24 hours to record their dry weights. STATISTIX 8 was used for data analysis (Analytical Software, Tallahassee, FL, USA). Means were compared by using Tukey's HSD test at $P < 0.05$.

3. Results

3.1. Nodulation Regulatory (*Rj*) Genes of Myanmar Soybean Cultivars Identified by an Inoculation Test

Identifying the *Rj* genes of soybean varieties is important to determine their host specificity and compatibility with specific bradyrhizobia. We evaluated the *Rj* genotypes of soybean cultivars from Myanmar to identify their nodulation *Rj* genes and estimate their compatibility with strains to be inoculated. Among the tested cultivars, six (Shan Seine [local], Hinthada [local], Madaya [local], Yezin-3, Yezin-7 and Yezin-11) were identified as harboring the Rj_4-gene. Only two cultivars, Yezin-6, Yezin-8, were classified as *non-Rj*-gene harboring cultivars. Yezin-9 and Yezin-10 were identified as Rj_3- and Rj_2Rj_3-gene harboring cultivars, respectively. The results of inoculation testing are shown in **Table 3**.

3.2. Nodulation *Rj* Genes of Myanmar Soybean Cultivars Identified by PCR Analysis

Among the tested cultivars, six (Shan Seine [local], Hinthada [local], Madaya [local], Yezin-3, Yezin-7 and Yezin-11) harbored Rj_4 gene alleles. Yezin-6, Yezin-8 and Yezin-9 did not harbor Rj_4 or Rj_2 genes, although Yezin-6 and Yezin-8 harbored the recessive alleles rj_4 and rj_2, and Yezin-9 and Yezion-10 harbored the recessive allele rj_4. We found the Rj_2 gene in Yezin-10. The results from the inoculation test and PCR analysis are shown in **Figure 1**.

3.3. Symbiotic Effectiveness of USDA 110 on Myanmar Soybean Cultivars

The number of nodules produced was significantly different when inoculated with *B. japonicum* USDA 110 (**Table 4**). The numbers of nodules ranged from 5 to 13 per plant. The most nodules were obtained from Yezin-10 (Rj_2Rj_3), but it was not significantly different from other cultivars, except for Madaya Local (Rj_4), which

Table 3. Nodulation regulatory genes (*Rj* genes) of cultivars.

Cultivar	Nodule No. plant^{-1} on inoculated strains			*Rj* gene
	USDA 33	Is-1	Is-34	
Shan Seine	Medium	Medium	None	Rj_4
Madaya	Medium	Medium	Low	Rj_4
Hinthada	Medium	High	None	Rj_4
Yezin-3	Medium	High	Low	Rj_4
Yezin-6	Medium	Medium	Medium	*non-Rj*
Yezin-7	Medium	High	None	Rj_4
Yezin-8	Medium	Medium	Medium	*non-Rj*
Yezin-9	Low	Medium	Medium	Rj_3
Yezin-10	None	None	High	Rj_2Rj_3
Yezin-11	Medium	Medium	None	Rj_4

High = 10 - 15 nodules plant^{-1}; Medium = 4 - 9 nodules plant^{-1}; Low = 1 - 3 nodules plant^{-1}; None = No nodulation. This division was based on Htwe *et al.* [43].

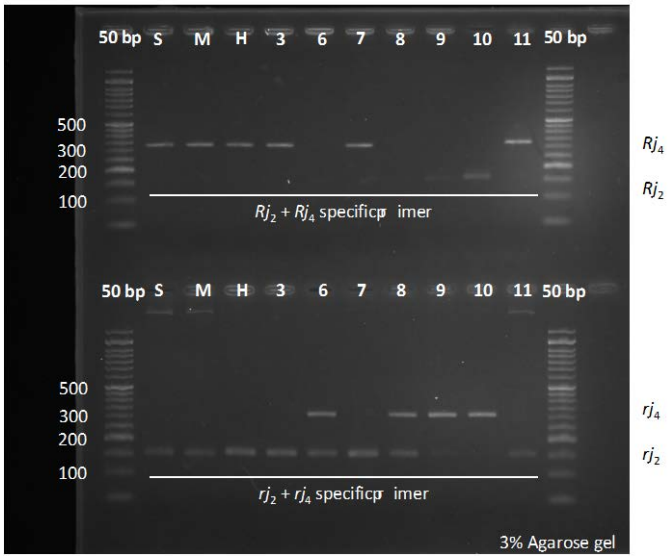


Figure 1. Expression of *rj* or *Rj* gene alleles amplified by multiplex PCR with Rj_2 and Rj_4, and rj_2 and rj_4 specific primers. S: Shan Seine; M: Madaya; H: Hinthada; 3: Yezin-3; 6: Yezin-6; 7: Yezin-7; 8: Yezin-8; 9: Yezin-9; 10: Yezin-10; 11: Yezin-11.

Table 4. Effect of *B. japonicum* USDA 110 strain on acetylene reduction activity, nodulation and plant growth of Myanmar soybean cultivars at 28 DAS.

Cultivars	NN (No. plant^{-1})	NDW (mg·plant^{-1})	SDW (g·plant^{-1})	RDW (g·plant^{-1})	ARA (μmol C_2H_4 h^{-1} plant^{-1})
Yezin-3	10.67 ab	15.70 ab	0.22 ab	0.09 cde	0.49 ab
Yezin-6	9.67 ab	15.40 ab	0.23 ab	0.13 ab	0.52 ab
Yezin-7	10.67 ab	12.60 ab	0.16 bc	0.08 de	0.35 ab
Yezin-8	9.00 ab	19.20 a	0.23 ab	0.11 bcd	0.51 ab
Yezin-9	5.33 ab	11.40 ab	0.21 ab	0.12 abc	0.58 ab
Yezin-10	13.00 a	10.00 ab	0.20 ab	0.11 bcd	0.43 ab
Yezin-11	12.00 ab	23.80 a	0.29 a	0.15 a	0.65 a
Shane Seine	8.00 ab	11.80 ab	0.14 bc	0.08 de	0.11 ab
Hinthada	10.67 ab	16.30 ab	0.27 a	0.12 abc	0.41 ab
Madaya	5.00 b	2.80 b	0.09 c	0.05 e	0.08 b

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. Yezin-6 was used as control. Nodule number, nodule dry weight and ARA value for control were zero. Shoot and root dry weight of control was 0.20 and 0.12 g·plant^{-1}, respectively.

produced the fewest nodules. Nodule dry weights also differed significantly among the cultivars. The nodule dry weights of Yezin-11 (Rj_4) and Yezin-8 (*non-Rj*) were greater than weights of the other cultivars but, with the exception of Madaya Local (Rj_4), these differences were not statistically significant.

Shoot dry weights were significantly different between some of the cultivars. The highest shoot biomass (0.29 g·plant^{-1}) was obtained from Yezin-11 (Rj_4), but this did not differ significantly from that of Yezin-3, Yezin-6, Yezin-8, Yezin-9, Yezin-10, or Hinthada local cultivars. Significantly greater root dry weight was recorded for Yezin-11 (Rj_4), but it was not significantly different from Yezin-6, Yezin-9, or Hinthada local cultivars. The nitrogenase activities varied significant among soybean cultivars when inoculated with *B. japonicum* USDA110 (**Table 4**). The highest ARA values were obtained from Yezin-11 (Rj_4), at 0.65 μmol·h^{-1}·plant^{-1}, but this did not differ significantly from that of the other cultivars, except for the Madaya local cultivar with the lowest nitrogenase activity. These results indicated that nodulation, N fixation, and plant growth of soybean cultivars differed

when inoculated with *B. japonicum* USDA110. Higher N-fixing cultivars, in terms of ARA per plant, were Yezin-11 (Rj_4), Yezin-9 (Rj_3), Yezin-6 (*non-Rj*), Yezin-8 (*non-Rj*), Yezin-3 (Rj_4), and Yezin-10 (Rj_2Rj_3). These top six cultivars, with higher N fixing potential, were selected for the next experiment.

3.4. Symbiotic Effectiveness of USDA 110 on Selected Soybean Cultivars

The number of nodules, ranging from 10 to 17.67 per plant, differed significantly among cultivars when inoculated with *B. japonicum* USDA 110 (**Figure 2(a)**). We observed a significant increase in the number of nodules in Yezin-11, followed by Yezin-10. Nodule dry weight was also significantly different among treatments, due to inoculation with *B. japonicum* USDA 110 (**Figure 2(b)**). The highest nodule dry weight was observed for Yezin-11, but it was not statistically different from that of other cultivars, except for Yezin-10 with the lowest nodule dry weight. Shoot dry weights varied significantly among cultivars, ranging from 0.45 to 0.63 g·plant^{-1} (**Figure 3(a)**). The highest shoot dry weight was obtained for Yezin-11 (Rj_4), but it was not statistically different from that of other cultivars, except for Yezin-6 (*non-Rj*). Root dry weight differed significantly among the cultivars (**Figure 3(b)**). The highest root dry weight was obtained from Yezin-11 (Rj_4). There were significant differences between the cultivars in nitrogenase activity, which ranged from 0.36 to 1.49 μmol·h^{-1}·plant^{-1} (**Figure 4**). The highest ARA value was obtained from Yezin-11 (Rj_4). Although the lowest ARA value was obtained from Yezin-8 (*non-Rj*), it did not differ statistically from Yezin-3 (Rj_4), Yezin-6 (*non-Rj*), Yezin-9 (Rj_3), or Yezin-10 (Rj_2Rj_3). These results indicated that Yezin-11 (Rj_4) was the most efficient cultivar, with the most nodules, the highest nodule, shoot, and root dry weights, and the greatest nitrogenase activity.

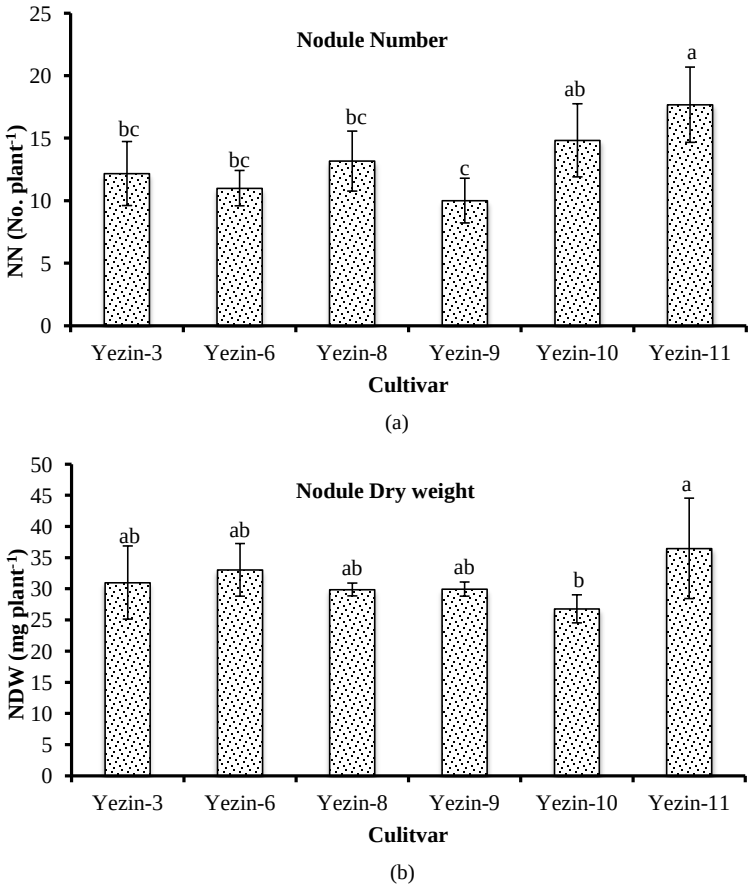
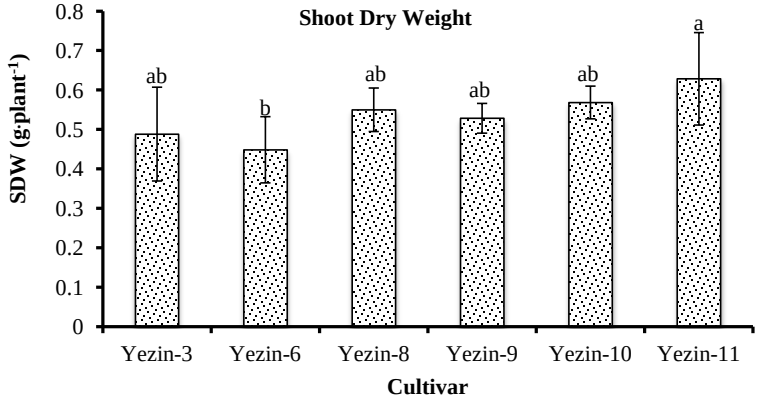
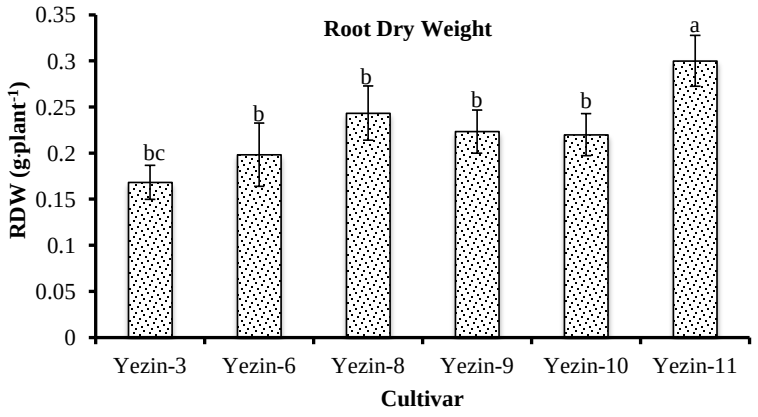


Figure 2. Effect of *B. japonicum* USDA 110 strain on (a) Nodule number and (b) Nodule dry weight of selected Myanmar soybean cultivars at 28 DAS. Mean values followed by same letters are not significantly different at $P > 0.05$ (Tukey's test). Yezin-6 and Yezin-8 were used as control treatments. Nodule number and nodule dry weight for controls were zero.



(a)



(b)

Figure 3. Effect of *B. japonicum* USDA 110 strain on (a) Shoot dry weight (SDW) and (b) Root dry weight of selected Myanmar soybean cultivars at 28 DAS. Mean values followed by same letters are not significantly different at $P > 0.05$ (Tukey's test). Yezin-6 and Yezin-8 was used as control treatments. Shoot and root dry weight of controls were 0.37 and 0.19 g·plant^{-1}, and 0.46 and 0.22 g·plant^{-1}, respectively.

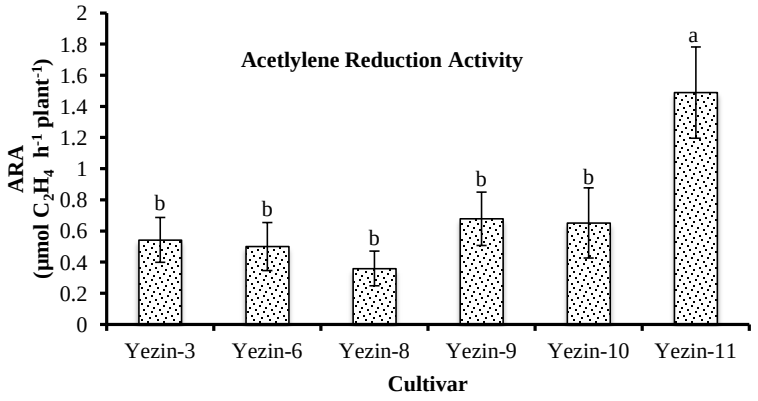


Figure 4. Effect of *B. japonicum* USDA 110 strain on Acetylene reduction activity (ARA) of Selected Myanmar soybean cultivars at 28 DAS. Mean values followed by same letters are not significantly different at $P > 0.05$ (Tukey's test). Yezin-6 and Yezin-8 were used as control treatments. ARAs for each control were zero.

4. Discussion

Symbiotic N fixation of soybean could provide 65 to more than 160 kg fixed N·ha^{-1} [30], accounting for 40 to 70% of the total N requirement. This symbiosis is highly specific, as a particular species or strain of *Rhizobia* could induce a symbiotic association with only a specific leguminous species or cultivars [31]. This specificity involves molecular recognition between the host and the bacterium, through exchange of compound signals that induce nodulation and N fixation [32] [33]. A *Rhizobium* strain that is effective on one legume might not be highly effective on other legumes [34], as the host legume has a dominant role in determining the nodule forming strain. Saeki *et al.* [17] stated that *Rj* genotypes of soybean cultivars have the ability to affect both compatibility and preference for nodulation between the host cultivar and soybean *Rhizobia*.

Compatibility between *Rj* soybean genotypes and soybean-nodulating bradyrhizobia must be considered in selecting the best varieties and strains for soybean cultivation. Therefore, nodulation *Rj* genes collected from soybean cultivars in Myanmar were evaluated to determine compatibility and preference between strains and cultivars. The *Rj* genes could be identified by inoculating with specific strains of *Bradyrhizobium*, such as *B. japonicum* Is-1, *B. elkanii* USDA33 and *B. japonicum* Is-34 [13]. The strains Is-1, USDA33, and Is-34 failed to form nodules on the roots of soybean cultivars harboring the Rj_2Rj_3, Rj_3 and Rj_4 genes, respectively [10] [27]. In this study, Yezin-6 and Yezin-8 formed nodules with all inoculated strains. Therefore, we identified them as non-*Rj*-gene harboring cultivars. The soybean cultivars Shan Seine (local), Hinthada (local), Yezin-7 and Yezin-11 were restricted in nodule formation when inoculated with *B. japonicum* Is-34. Therefore, we classified them as Rj_4 genotype cultivars. In the inoculation test, a few nodules were produced on the roots of Yezin-3 and Madaya (local) when inoculated with Is-34, but we assumed that they harbored the Rj_4 gene. One or two nodules were formed on the roots of Yezin-9 and D51 when inoculated with USDA33. Yamakawa *et al.* [22] stated that a few effective nodules and numerous ineffective nodules were produced by D51 when inoculated with USDA33. This agrees with our findings. Therefore, we assumed that Yezin-9 was an Rj_3 gene-harboring cultivar. Soe *et al.* [19] identified the *Rj* genes of some Myanmar cultivars. They reported that Hinthada, Southern Shan local, Northern Shan local, Yezin-3, and Yezin-11 cultivars harbored the Rj_4-gene, whereas Shan Sein, Shan Wha, Yezin-6, Yezin-8, and Yezin-14 cultivars did not harbor *Rj* genes.

In this study, we performed PCR analysis to confirm the *Rj* genotypes. Although we could not identify Rj_3, the analysis was very useful in identifying the Rj_4 and Rj_2 alleles. The cultivars Shan Seine (local), Hinthada (local), Madaya (local), Yezin-3, Yezin-7 and Yezin-11 harbored Rj_4 alleles. Yezin-6, Yezin-8, and Yezin-9 cultivars did not harbor other dominant *Rj* alleles, although they harbored the recessive alleles rj_2 and rj_4. Only Yezin-9 was identified as an Rj_2-gene harboring cultivar. Based on PCR analysis, we confirmed that Yezin-3 and Madaya (local) were Rj_4-genotype soybean cultivars, although they could also form a few nodules with their nodulation restricting strain Is-34. Contrary to Soe *et al.* [19], the *Rj* genotypes of Hinthada (local), Yezin-3, Yezin-6, Yezin-8 and Yezin-11 were the same and the results for Shan Seine (local) differed from those reported by Soe *et al.* [19]. In this study, Shan Seine (local) was identified as an Rj_4-gene harboring cultivar, according to the inoculation test and PCR analysis results. Therefore, PCR analysis for *Rj* gene determination was deemed necessary if the inoculation test results were uncertain.

According to the inoculation test and PCR results, the cultivars Shan Seine (local), Hinthada (local), Madaya (local), Yezin-3, Yezin-7 and Yezin-11 were identified as Rj_4-genotype cultivars. Yezin-6 and Yezin-8 were identified as non-*Rj* genotype cultivars. Yezin-9 and Yezin-10 were identified as harboring the Rj_3 and Rj_2Rj_3 genes, respectively. In Myanmar, the Rj_4 genotype cultivars were the most widely grown cultivars, accounting for 60% of the tested cultivars. Devine and Kuykendall [35] reported the Rj_4 genotype cultivars as most frequently found in Southeast Asia, but not common in North Asia. Devine and Breithaupt [36] also reported that 60% of the cultivars from Southeast Asia and 71.2% of those from Myanmar harbored Rj_4 genes.

In Myanmar, DAR has developed improved soybean cultivars to replace local cultivars. However, some Myanmar farmers have continued to grow local soybean cultivars, such as Shan Seine (local), Madaya (local) and Hinthada (local). Proper matching of soybean cultivars and *Rhizobia* strains optimizes performance through enhanced N fixation. In this study, we screened improved and local cultivars using the strain *B. japonicum* USDA110, as several studies have reported significant increases in growth, yield, nodulation, and N fixation of Myanmar soybean cultivars due to inoculation with this strain [20] [37] [38].

We found that local cultivars such as Madaya (local) (Rj_4), Shan Seine (local) (Rj_4) and Yezin-7 (Rj_4) showed lower nitrogenase activity, nodulation and plant growth. Hinthada (local) produced an increased number of no-

dules and nodule dry weight, but its N fixation was lower than other improved cultivars such as Yezin-11 and Yezin-3, despite having the Rj_4 genes in common. Soe *et al.* [20] also reported that improved Yezin cultivars were more efficient for N fixation compared with local cultivars. We also discovered that the improved Yezin cultivars Yezin-11 (Rj_4), Yezin-9 (Rj_3), Yezin-6 (*non-Rj*), Yezin-8 (*non-Rj*), Yezin-3 (Rj_4), and Yezin-10 (Rj_2Rj_3) were superior in N fixing capacity. These top six cultivars, showing higher nitrogenase activity, were selected for the second screening experiment. Selection of cultivars was based on nitrogenase activity and nodulation. Wani *et al.* [39] stated that the number of nodules or nitrogenase activity were genotypically variable within grain legume species. A legume plant with effective nodules could meet not only its own N requirements, but also enrich soil N content, thus improving soil fertility and sustainability [40].

In results from the second screening experiment, Yezin-11 (Rj_4) was the most efficient cultivar, with the most nodules, and highest nodule, shoot and root dry weights, and the greatest nitrogenase activity. When we compared the N fixing rates, in terms of ARA per plant inoculated with *B. japonicum* USDA 110, Yezin-9 (Rj_3) and Yezin-10 (Rj_2Rj_3) were most efficient in N fixation, though they did not differ significantly from the N fixation of Yezin-6 (*non-Rj*), Yezin-8 (*non-Rj*) and Yezin-3 (Rj_4). This might be related to the *Rj* genes, which can affect both preference and compatibility for nodulation between the host cultivar and soybean *Rhizobia* [13] [14] [17]. Yamakawa *et al.* [22] [23] stated that $Rj_2Rj_3Rj_4$-gene harboring cultivars had improved N fixation compared with the *non-Rj*, Rj_2, Rj_2Rj_3 and Rj_4 soybean cultivars when inoculated with *B. japonicum* USDA110. In both experiments, the Rj_4-gene harboring Yezin-11 cultivars showed higher N fixation capacity, followed by the Rj_3-gene harboring Yezin-9 cultivars. The N fixation activity and the ratio of N fixed from the atmosphere to the total N accumulation in plants vary significantly within soybean cultivars [41] [42].

5. Conclusion

Most Myanmar soybean cultivars were identified as harboring the Rj_4 gene. A few cultivars were classified as *non-Rj*, Rj_2Rj_3 and Rj_3 gene harboring. This was the first report of Rj_2Rj_3 and Rj_3 genotype soybean cultivars in Myanmar. We evaluated the N fixation and nodulation of Myanmar soybean cultivars by using the standard strain *B. japonicum* USDA 110. In both experiments, Yezin-11 (Rj_4) was the most efficient for N fixation and nodulation. It appeared that Yezin-11 (Rj_4) was more compatible with *B. japonicum* USDA110 based on the results from both experiments. Yezin-11 had about two to three times higher symbiotic N fixation capacity than the other soybean cultivars. Our study provides useful information for breeders seeking to produce cultivars efficiently at N fixation. Further study is needed on the effectiveness of different *Rj* genotypes with indigenous bradyrhizobia to increase soybean productivity via symbiotic N fixation.

Acknowledgements

This work was supported by Ministry of Education, Culture, Sports, Sciences and Technology of Japan. We are grateful to Dr. Htun Shwe (Researcher, Food Legume Section, Department of Agricultural Research) for providing soybean seeds.

References

[1] MOAI (2013) Myanmar Agriculture in Brief. Ministry of Agriculture and Irrigation, Naypyitaw.

[2] CSO (2006) Myanmar Agricultural Statistics (1992-1993 to 2004-2005). Central Statistical Organization, Ministry of National Planning and Economic Development, Yangon.

[3] Unkivich, M.J. and Pate, J.S. (2000) An Appraisal of Recent Field Measurements of Symbiotic N_2 Fixation by Annual Legumes. *Field Crop Research*, **65**, 211-228. http://dx.doi.org/10.1016/S0378-4290(99)00088-X

[4] van Kessel, C. and Hartley, C. (2000) Agricultural Management of Grain Legumes: Has It Led to An Increased in Nitrogen Fixation? *Field Crop Research*, **65**, 165-181. http://dx.doi.org/10.1016/S0378-4290(99)00085-4

[5] Van, K., Kim, M.Y. and Lee, S.H. (2007) Genomic of Root Nodulation. *Genomic-Assisted Crop Improvement*, **2**, 435-452. http://dx.doi.org/10.1007/978-1-4020-6297-1_16

[6] Hayashi, M., Saeki, Y., Haga, M., Harada, K., Kouchi, H. and Umehara, Y. (2012) A Review: *Rj* (*rj*) Genes Involved in Nitrogen-Fixing Root Nodule Formation in Soybean. *Breeding Science*, **61**, 544-553. http://dx.doi.org/10.1270/jsbbs.61.544

[7] Williams, L.F. and Lynch, D.L. (1954) Inheritance of a Non-Nodulating Character in the Soybean. *Agronomy Journal*, **46**, 28-29. http://dx.doi.org/10.2134/agronj1954.00021962004600010008x

[8] Caldwell, B.E. (1966) Inheritance of a Strain-Specific Ineffective Nodulation in Soybeans. *Crop Science*, **6**,427-428. http://dx.doi.org/10.2135/cropsci1966.0011183X000600050010x

[9] Caldwell, B.E., Hinson, K. and Johnson, H.W. (1966) A Strain-Specific Ineffective Nodulation Reaction in the Soybean *Glycine max* L. Merrill. *Crop Science*, **6**, 495-496. http://dx.doi.org/10.2135/cropsci1966.0011183X000600050033x

[10] Vest, G. (1970) *Rj*$_3$-A Gene Conditioning Ineffective Nodulation. *Crop Science*, **10**, 34-35. http://dx.doi.org/10.2135/cropsci1970.0011183X001000010013x

[11] Vest, G. and Caldwell, B.E. (1972) *Rj*$_4$—A Gene Conditioning Ineffective Nodulation in Soybean. *Crop Science*, **12**, 692-693. http://dx.doi.org/10.2135/cropsci1972.0011183X001200050042x

[12] Trese, A.T. (1995) A Single Dominant Gene in McCall Soybean Prevents Effective Nodulation with *Rhizobium fredii* USDA257. *Euphytica*, **81**, 279-282. http://dx.doi.org/10.1007/BF00025618

[13] Ishizuka, J., Suemasu, Y. and Mizogami, K. (1991) Preference of *Rj*-Soybean Cultivars for *Bradyrhizobium japonicum* for Nodulation. *Soil Science and Plant Nutrition*, **37**, 15-21. http://dx.doi.org/10.1080/00380768.1991.10415005

[14] Ishizuka, J., Yokoyama, A. and Suemasu, Y. (1991) Relationship between Serotypes of *Bradyrhizobium japonicum* and Their Compatibility with *Rj*-Cultivars for Nodulation. *Soil Science and Plant Nutrition*, **37**, 23-30. http://dx.doi.org/10.1080/00380768.1991.10415006

[15] Minami, M., Yamakawa, T., Yamamoto, A., Akaso, S. and Saeki, Y. (2009) Estimation of Nodulation Tendency among *Rj*-Genotype Soybeans Using Bradyrhizobial Community Isolated from an Andosol. *Soil Science and Plant Nutrition*, **55**, 65-72. http://dx.doi.org/10.1111/j.1747-0765.2008.00333.x

[16] Saeki, Y., Akagi, I., Takaki, H. and Nagatomo, Y. (2000) Diversity of Indigenous *Bradyrhizobium* Strains Isolated from Three Different *Rj*-Soybean Cultivars in Terms of Randomly Amplified Polymorphic DNA and Intrinsic Antibiotic Resistance. *Soil Science and Plant Nutrition*, **46**, 917-926. http://dx.doi.org/10.1080/00380768.2000.10409157

[17] Saeki, Y., Kaneko, A., Hara, T., Suzuki, K., Yamakawa, T., Nguyen, M.T., Nagatomo, Y. and Akao, S. (2005) Phylogenetic Analysis of Soybean Nodulating Rhizobia Isolated from Alkaline Soils in Vietnam. *Soil Science and Plant Nutrition*, **51**, 1043-1052. http://dx.doi.org/10.1111/j.1747-0765.2005.tb00143.x

[18] Saeki, Y., Minami, M., Yamamoto, A. and Akao, S. (2008) Estimation of the Bacterial Community Diversity of Soybean-Nodulating Bradyrhizobia Isolated from *Rj*-Genotype Soybeans. *Soil Science and Plant Nutrition*, **54**, 718-724. http://dx.doi.org/10.1111/j.1747-0765.2008.00300.x

[19] Soe, K.M., Yamakawa, T., Hashimoto, S. and Sarr, P. (2013) Phylogenetic Diversity of Indigenous Soybean Bradyrhizobia from Different Agro-Climatic Regions in Myanmar. *Science Asia*, **39**, 574-583. http://dx.doi.org/10.2306/scienceasia1513-1874.2013.39.574

[20] Soe, K.M. and Yamakawa, T. (2013) Evaluation of Effective Myanmar *Bradyrhizobium* Strains Isolated from Myanmar Soybean and Effects of Coinoculation with *Streptomyces griseoflavus* P4 for N Fixation. *Soil Science and Plant Nutrition*, **59**, 361-370. http://dx.doi.org/10.1080/00380768.2013.794437

[21] Soe, K.M. and Yamakawa, T. (2013) Low-Density Co-Inoculation of Myanmar *Bradyrhizobium yuyanmingense* MAS34 and *Streptomyces griseoflavus* P4 to Enhance Symbiosis and Seed Yield in Soybean Varieties. *American Journal of Plant Science*, **4**, 1879-1892. http://dx.doi.org/10.4236/ajps.2013.49231

[22] Yamakawa, T., Eriguchi, M., Hussain, A.K.M.A. and Ishizuka, J. (1999) Soybean Preference for *Bradyrhizobium japonicum* for Nodulation: Nodulation by *Rj*$_2$*Rj*$_3$*Rj*$_4$-Genotypes Isolated from the Progenies of a Cross between Soybean cv. ICA-2 (*Rj*$_2$*Rj*$_3$) and Hill (*Rj*$_4$). *Soil Science and Plant Nutrition*, **45**, 461-469. http://dx.doi.org/10.1080/00380768.1999.10409360

[23] Yamakawa, T., Hussain, A.K.M.A. and Ishizuka, J. (2003) Soybean Preference for *Bradyrhizobium japonicum* for Nodulation Occupation of Serogroup USDA110 in Nodules of Soybean Plants Harboring Various *Rj*-Genes Grown in a Field. *Soil Science and Plant Nutrition*, **49**, 835-841. http://dx.doi.org/10.1080/00380768.2003.10410345

[24] Yang, S., Tang, F., Gao, M., Krishnan, H.B. and Zhu, H. (2010) R Gene-Controlled Host Specificity in the Legume-Rhizobia Symbiosis. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 18735-18740. http://dx.doi.org/10.1073/pnas.1011957107

[25] Tang, F., Yang, S., Liu, J., Gao, M. and Zhu, H. (2014) Fine Mapping of the *Rj*$_4$ Locus, a Gene Controlling Nodulation Specificity in Soybean. *Molecular Breeding*, **33**, 691-700. http://dx.doi.org/10.1007/s11032-013-9985-y

[26] Hayashi, M., Shiro, S., Kanamori, H., Mori-Hosokawa, S., Sasaki-Yamagata, H., Sayama, T., Nishioka, M., Takahashi, M., Ishimoto, M., Kataoyose, Y., Kaga, A., Harada, K., Kouchi, H., Saeki, Y. and Umehara, Y. (2014) A Thaumatin-Like Protein, *Rj*$_4$, Controls Nodule Symbiotic Specificity in Soybean. *Plant Cell Physiology*, **55**, 1679-1689. http://dx.doi.org/10.1093/pcp/pcu099

[27] Ishizuka, J., Kim, S.D., Hussain, A.K.M.A. and Yamakawa, T. (1993) Soybean Preference for *Bradyrhizobium japonicum* for Nodulation: Isolation of *Rj*$_2$*Rj*$_4$-Lines from the Cross of Soybean cvs. IAC-2 (*Rj*$_2$) and Hill (*Rj*$_4$). *Soil Science*

and Plant Nutrition, **39**, 79-86. http://dx.doi.org/10.1080/00380768.1993.10416977

[28] Nakano, Y., Yamakawa, T., Ikeda, M. and Ishizuka, J. (1997) Nodulation of *Rj*-Soybean Varieties with *Rhizobium fredii* USDA193 under Limited Supply of Nutrients. *Soil Science and Plant Nutrition*, **43**, 929-932. http://dx.doi.org/10.1080/00380768.1997.10414659

[29] Kuykendall, L.D. (1979) Transfer of R Factors to and between Genetically Marked Sublines of *Rhizobium japonicum*. *Applied Environmental Microbiology*, **37**, 862-866.

[30] Klubeck, B.P., Hendrickson, L.L., Zablotowicz, R.M., Skwara, J.E., Varsa, E.C., Smith, S., Isleib, T.G., Maya, J., Valdes, M., Dazzo, F.B., Todd, R.L. and Walgenback, D.D. (1988) Competitiveness of Selected *Bradyrhizobium japonicum* Strains in Midwestern USA Soils. *Soil Science Society American Journal*, **52**, 662-666. http://dx.doi.org/10.1080/00380768.1997.10414659

[31] Somasegaran, P. and Hoben, H.J. (1994) Handbook for Rhizobia: Methods in Legumes-Rhizobium Technology. Springer-Verlag Inc, New York, 450. http://dx.doi.org/10.1007/978-1-4613-8375-8

[32] Denarie, J., Debelle, E. and Rosenberg, C. (1992) Signalling and Host Range Variation in Nodulation. *Annual Review Microbiology*, **46**, 446-453. http://dx.doi.org/10.1146/annurev.mi.46.100192.002433

[33] Perret, X., Staechelin, C. and Brouthton, W.J. (2000) Molecular Basis of Symbiotic Promiscuity. *Microbiology and Molecular Biology Reviews*, **64**, 180-201. http://dx.doi.org/10.1128/MMBR.64.1.180-201.2000

[34] Boonkerd, N. and Singleton, P. (2002) Production of *Rhizobium* Biofertilizer. In: Kannaiyan, S., Ed., *Biotechnology of Biofertilizers*, Narosa Publishing House, New Delhi, 122-128.

[35] Devine, T.E. and Kuykendall, L.D. (1996) Host Genetic Control of Symbiosis in Soybean (*Glycine max* L.). *Plant Soil*, **186**, 173-187. http://dx.doi.org/10.1007/BF00035072

[36] Devine, T.E. and Breithaupt, B.H. (1981) Frequencies of Nodulation Response Alleles, Rj_2 and Rj_4 in Soybean Plant Introductions and Breeding Lines. USDA Technical Bulletin No. 2618.

[37] Soe, K.M., Bhromsiri, A., Karladee, D. and Yamakawa, T. (2012) Effects of *Endophytic actinomycetes* and *Bradyrhizobium japonicum* Strains on Growth, Nodulation, Nitrogen Fixation and Seed Weight of Different Soybean Varieties. *Soil Science and Plant Nutrition*, **58**, 319-325. http://dx.doi.org/10.1080/00380768.2012.682044

[38] Aung, T.T., Tittaburt, P., Boonkerd, N. and Herridge, D. (2013) Co-Inoculation Effects of *Bradrhizobium japonicum* and *Azospirillum* sp. on Competitive Nodulation and Rhizosphere Eubacterial Community Structures of Soybean under Rhizobia-Established Soil Conditions. *African Journal of Biotechnology*, **12**, 2850-2862.

[39] Wani, S.P., Rupela, O.P. and Lee, K.K. (1995) Sustainable Agriculture in a Semiarid Tropic through Biological Nitrogen Fixation in Grain Legume. *Plant Soil*, **174**, 29-49. http://dx.doi.org/10.1007/BF00032240

[40] Thabgaraju, M. and Werner, D. (2004) Indigenous Strains of Rhizobia and Their Performance in Specific Regions of India. In: Werner, D., Ed., *Biological Resources and Migration*, Springer-Verlag, Berlin, 174. http://dx.doi.org/10.1007/978-3-662-06083-4_17

[41] Hungria, M. and Bohrer, T.R.J. (2000) Variability of Nodulation and Dinitrogen Fixation Capacity among Soybean Cultivars. *Biology and Fertility of Soils*, **31**, 45-52. http://dx.doi.org/10.1007/s003740050622

[42] Nohara, T., Nakayama, N., Nakamura, T., Takahashi, M., Maruyama, S., Arihara, J. and Shimada, S. (2006) Cultivar Differences of Nitrogen Fixation Capacity and Its Contribution to Nitrogen Accumulation in Soybean Grown in the Field with a High Soil Nitrate Level. *Japanese Journal of Crop Science*, **75**, 350-359. http://dx.doi.org/10.1626/jcs.75.350

[43] Htwe, A.Z., Yamakawa, T., Sarr, P.S. and Sakata, T. (2015) Diversity and Distribution of Soybean-Nodulation Bradyrhizobia Isolated from Major Soybean-Growing Regions in Myanmar. *African Journal of Microbiology Research*, **9**, 2183-2196. http://www.academicjournals.org/journal/AJMR/article-full-text-pdf/C58126E55962