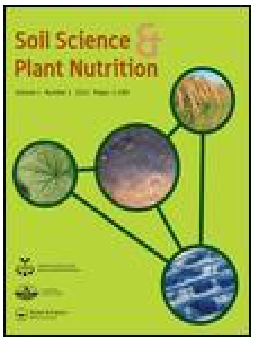

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Searching for Myanmar indigenous *Bradyrhizobium* type C strains that best identify Rj_4 genotypes in soybean

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ABSTRACT

Bradyrhizobium species are symbiotic partners of soybean plants. However, some *Bradyrhizobium* bacteria do not form functional nodules on the roots of Rj_4 genotype soybean cultivars. Our objective was to identify the strains of *Bradyrhizobium* (i.e., type C strains) that are least competent to form nodules on the roots of this plant genotype. We checked (i) previously isolated type C strains of Myanmar *Bradyrhizobium elkanii* (MMY6-1, MMY6-2, and MMY6-5), (ii) previously isolated type C strains of Myanmarese *Bradyrhizobium* spp. (MMY3-5 and MMY3-7), and (iii) strain Is-34 of *B. japonicum*, for nodule formation when associated with Rj_4 and other *Rj* genotype soybeans. Strains in groups (i), (ii), and (iii) are known to be incompatible with Rj_4 soybean genotypes. MMY3-5 and MMY3-7 produced functional nodules when associated with Rj_4 and other *Rj* genotype soybean cultivars, except Hill (Rj_4) cultivar. The ratios of ineffective nodule numbers/total nodule numbers (*I/T* ratios) for MMY6-1, MMY6-2, MMY6-5, and Is-34 in association with Rj_4 soybean cultivars were > 0.5, demonstrating incompatibility between these bacterial strains and the Rj_4 genotype. Interestingly, the *I/T* ratios of MMY6-1 and MMY6-2 were higher than that of Is-34 in almost all Rj_4 soybean cultivars. Thus, the nodule-forming abilities of the *B. elkanii* strains MMY6-1 and MMY6-2 were strongly suppressed in Rj_4 soybean cultivars; these strains may therefore be useful to identify the Rj_4 genotype in soybean cultivars.

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Bradyrhizobium elkanii; incompatibility; nodulation; Rj_4; soybean

1. Introduction

The genus *Bradyrhizobium* is famous as symbiont of host soybean (*Glycine max* (L.) Merr.) plants. However, some strains of *Bradyrhizobium* inhibit the formation of functional root nodules, the organs in which atmospheric nitrogen is fixed. For example, *Bradyrhizobium* strains BTAi1 and ORS278 do not induce nodule formation on soybean roots, because these strains lack nod genes (Giraud *et al*. 2007). These nodulation incompatibilities arise because the *Rj* genotype in soybean influences compatibility between the symbionts, thereby inhibiting functional nodule formation on the host roots (Saeki *et al*. 2005; Hayashi *et al*. 2012). *Bradyrhizobium* strains have been classified into nodulation types A, B, and C based on degree of compatibility between bradyrhizobia and the *Rj* genotype in soybean cultivars (Ishizuka *et al*. 1991a, 1991b). The type A strains promote nodulation in all *Rj* genotype cultivars. Type B and C strains uniquely inhibit the formation of effective nodules in Rj_2Rj_3 and Rj_4 genotype cultivars, respectively. Types B and C preferentially form symbioses with Rj_4 and Rj_2Rj_3 soybean cultivars, respectively.

The *Rj* genotypes (non-*Rj*, rj_1, Rj_2, Rj_3, and Rj_4) occur in nature (Devine and Kuykendall 1996). Genotypes like $Rj_2Rj_3Rj_4$ can be constructed by crossing soybean cv. ICA-2 (Rj_2Rj_3) with Hill (Rj_4) (Yamakawa *et al*. 1999). Soe *et al*. (2013) reported the occurrence of soybean cultivars with non-*Rj* and Rj_4 genotypes in Myanmar. Cultivars with non-*Rj*, Rj_2Rj_3, and Rj_4 genotypes have also been reported in other studies (Htwe *et al*. 2015b). Rj_4 genotype soybean cultivars are widely grown in Myanmar, where they account for 60% of all production (Htwe *et al*. 2015b). More than 60% of soybeans in South East Asia are reported to have the Rj_4 genotype (Devine and Kuykendall 1996).

The *Rj* genotypes have known to be usually identified by their inoculation properties (Ishizuka *et al*. 1993). Strains Is-1, USDA33, and Is-34 are useful for identifying *Rj* genotypes because they uniquely inhibit nodulation of the host genotypes, i.e., Rj_2Rj_3, Rj_3, and Rj_4, respectively (Vest 1970; Ishizuka *et al*. 1993). The identification of *Rj* genotypes with the inoculation test is convenient, inexpensive, and simple to perform. However, we reported in our previous study that the Is-34 (type C) strain forms a few nodules on the roots of some Rj_4 soybean cultivars (Htwe *et al*. 2015a). Thus, bacterial strains that strongly inhibit nodule formation in Rj_4 genotype cultivars should be replaced with Is-34 (type C) for the purpose of identifying Rj_4 genotypes in soybean cultivars.

The type C strain has rarely been isolated in Myanmar. Among 43 indigenous isolates, Soe *et al*. (2013) identified *B. yuanmingense* MAS28 as a type C strain. In our previous study of 120 isolates (Htwe *et al*. 2015a), we identified five type C strains: *Bradyrhizobium* spp. strains MMY3-5 and MMY3-7, and *B. elkanii* strains MMY6-1, MMY6-2, and MMY6-5. Identifying *Bradyrhizobium* type C strains that are incompatible with Rj_4 genotype soybean cultivars is important for the

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detection of Rj_4 genotypes in host plants. Nodule formation by *Bradyrhizobium* type C strains is restricted in Rj_4 genotype cultivars; hence, type C strains are especially useful for identifying the Rj_4 genotype (Ishizuka *et al.* 1991a, 1991b).

In Myanmar, many researchers have been emphasizing on selection of strain to increase nitrogen fixation of soybean. Recently, Department of Agricultural Research (DAR) has released improved varieties such as Yezin-9, Yezin-10, and Yezin-11. However, identification of *Rj* genotypes of some released cultivars is still lack. To recommend the most efficient nitrogen-fixing cultivars, it is necessary to take into consideration on the evaluation of symbiotic effectiveness with inoculated strain and identification of nodulation regulatory genes of cultivar. Our previous identification of Rj_4 genotypes was based on inoculation results of Is-34. Four cultivars of soybean: Shan Sein (local), Hinthada (local), Yezin-7, and Yezin-11 was restricted nodule formation by *B. japonicum* Is-34, suggesting they were Rj_4 genotype cultivars. However, a few effective nodules were formed on the roots of other known Rj_4 genotype. Those results can cause the misclassification whether it harbored Rj_4 genotypes or not in soybean. Therefore, searching for type C strain with strong restriction ability for nodule formation, which can be used in identifying Rj_4 genotypes in soybean, is necessary to be able to replace Is-34. This study was conducted to search for the type C strains that best identify Rj_4 genotypes in soybean.

2. Materials and methods

2.1. Origins of the Bradyrhizobium strains

The *Bradyrhizobium japonicum* Is-34 (type C) strain was obtained from the Plant Nutrition Laboratory at Kyushu University, Japan. Myanmarese indigenous bradyrhizobia [*Bradyrhizobium* spp. strains MMY3-5 (type C) and MMY3-7 (type C), and *B. elkanii* strains MMY6-1 (type C), MMY6-2 (type C), and MMY6-1 (type C)] were isolated in Madaya, which is located in the Madalay Region of Myanmar. The nodulation types of these strains were identified in our previous study (Htwe *et al.* 2015a).

2.2. Origins of the soybean cultivars

Myanmar soybean cultivars were obtained from the Food Legume Section, Department of Agricultural Research, Yezin, Myanmar. The *Rj* genotypes were identified by Htwe *et al.* (2015b). Other cultivars were obtained from the Plant Nutrition Laboratory, Department of Bioresources and Bioenvironmental Sciences, Kyushu University. The *Rj* genotypes were described by Ishizuka *et al.* (1991a) and Yamakawa *et al.* (1999). The name of varieties and their *Rj* genotypes are listed in Table 1.

2.3. Cultivation, crop management, and data collection

Seeds were surface-sterilized by soaking them in 2.5% sodium hypochlorite solution for 5 min and 99.5% ethanol for 5 min, and rinsing them with five washes of sterilized, half-strength modified (nitrogen-free) Hoagland Nutrient (MHN) solution (Nakano *et al.* 1997). Seven seeds were sown in each prepared culture pot containing 1.0 L of vermiculite and 0.6 L of MHN solution. The *Bradyrhizobium* strains were cultured in A1E liquid medium (Kuykendall 1987) and incubated on a rotary shaker (100 rpm) at 30°C for 7 days. Inoculants were prepared by diluting 1.0 mL of liquid bacterial culture in 99 mL sterilized MHN solution to obtain bacterial suspensions containing 10^7 cells mL^{-1}. Each pot was inoculated with 35.0 mL of bacterial suspension immediately after the seven seeds were sown therein. Pots were maintained under controlled conditions with natural light for 4 weeks. Control pots without inoculate were used to check for contamination. The plants were watered weekly with autoclaved deionized water. After 4 weeks, the plants were checked to determine whether there were effective or ineffective nodules; our objective was to detect nodulation incompatibility with Rj_4 soybean cultivars. The experiment was conducted in the period between March and May 2018.

Table 1. Nodulation regulatory gene (*Rj*) of soybeans used in this study.

Cultivar	*Rj* gene
Yezin-6	Non-*Rj*
C244	$Rj_2Rj_3Rj_4$
CNS	Rj_2
D51	Rj_3
Fukuyutaka	Rj_4
Hill	Rj_4
Yezin-7	Rj_4
Yezin-11	Rj_4
Shan Seine	Rj_4
Hinthada	Rj_4

2.4. Statistical 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 taken to indicate statistical significance.

3. Results

Bradyrhizobium spp. strains MMY3-5 and MMY3-7, *B. elkanii* strains MMY6-1, MMY6-2, and MMY6-5, and *B. japonicum* Is-34 (control strain) were all fully competent nodule formers when associated with the Yezin-6 (non-*Rj*) soybean cultivar (Table 2). Is-34 and MMY6-1 formed significantly higher numbers of nodules than MMY3-5 and MMY3-7; however, the nodule-forming performances of Is-34 and MMY6-1 were not significantly different from those of MMY6-2 and MMY6-5. All of the bacterial strains were effective nodule formers when associated with CNS (Rj_2Rj_3). Nodule numbers on plants inoculated with MMY6-1, MMY6-2, and MMY6-5 were significantly higher than that on plants inoculated with other strains (Table 3). All strains of bacteria formed effective nodules on the D51 (Rj_3) soybean cultivar. Nodule numbers were significantly higher in plants inoculated with MMY6-1 and MMY6-2 than in plants inoculated with other strains (Table 4). Thus, all bacterial strains readily formed effective nodules when in association with Yezin-6 (non-*Rj*), CNS (Rj_2Rj_3), and D51 (Rj_3). No ineffective nodules developed in these bacterial/soybean combinations.

Bacterial strains MMY6-1, MMY6-2, MMY6-5, and Is-34 were not competent nodule formers when associated with C244 ($Rj_2 Rj_3Rj_4$) (Table 5). The numbers of effective nodules in plants inoculated with MMY6-1, MMY6-2, MMY6-5, and Is-34 were

Table 2. Detection for nodulation incompatibility of different *Bradyrhizobium* strains on Yezin-6 (non-*Rj*) soybean variety.

Variety	Isolate	Effective nodule (No. plant^{-1})	Ineffective nodule (No. plant^{-1})	Total nodule (No. plant^{-1})	I/T	Incompatibility
Yezin-6	Is-34	13.6 ± 2.5 a	0.0 ± 0.0	13.6 ± 2.5 a	0.00 ± 0.00	−
(non-*Rj*)	MMY6-1	13.0 ± 3.9 a	0.0 ± 0.0	13.0 ± 3.9 a	0.00 ± 0.00	−
	MMY6-2	12.8 ± 3.2 *ab*	0.0 ± 0.0	12.8 ± 3.2 *ab*	0.00 ± 0.00	−
	MMY6-5	11.2 ± 2.3 abc	0.0 ± 0.0	11.2 ± 2.3 abc	0.00 ± 0.00	−
	MMY3-5	9.6 ± 1.9 bc	0.0 ± 0.0	9.6 ± 1.9 bc	0.00 ± 0.00	−
	MMY3-7	7.8 ± 1.3 c	0.0 ± 0.0	7.8 ± 1.3 c	0.00 ± 0.00	−

For each cultivar, mean value ± SD in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). Total nodule numbers means the sum of effective and ineffective nodules. The number indicating in table is the mean of 12 plants for all cultivars. + or − show the plants have or do not have the restriction ability.

Table 3. Detection for nodulation incompatibility of different *Bradyrhizobium* strains on CNS (Rj_2Rj_3) soybean variety.

Variety	Isolate	Effective nodule (No. plant^{-1})	Ineffective nodule (No. plant^{-1})	Total nodule (No. plant^{-1})	I/T	Incompatibility
CNS	Is-34	12.3 ± 2.1 b	0.0 ± 0.0	12.3 ± 2.1 b	0.00 ± 0.00	−
(Rj_2)	MMY6-1	22.2 ± 7.7 a	0.0 ± 0.0	22.2 ± 7.7 a	0.00 ± 0.00	−
	MMY6-2	19.1 ± 4.6 a	0.0 ± 0.0	19.1 ± 4.6 a	0.00 ± 0.00	−
	MMY6-5	20.3 ± 4.9 a	0.0 ± 0.0	20.3 ± 4.9 a	0.00 ± 0.00	−
	MMY3-5	7.3 ± 1.2 b	0.0 ± 0.0	7.3 ± 1.2 b	0.00 ± 0.00	−
	MMY3-7	9.0 ± 1.4 b	0.0 ± 0.0	9.0 ± 1.4 b	0.00 ± 0.00	−

For each cultivar, mean value ± SD in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). Total nodule numbers means the sum of effective and ineffective nodules. The number indicating in table is the mean of 12 plants for all cultivars. + or − show the plants have or do not have the restriction ability.

Table 4. Detection for nodulation incompatibility of *Bradyrhizobium* strains on D51 (Rj_3) soybean variety.

Variety	Isolate	Effective nodule (No. plant^{-1})	Ineffective nodule (No. plant^{-1})	Total nodule (No. plant^{-1})	I/T	Incompatibility
D51	Is-34	11.3 ± 1.8 b	0.0 ± 0.0	11.3 ± 1.8 b	0.00 ± 0.00	−
(Rj_3)	MMY6-1	16.0 ± 3.4 a	0.0 ± 0.0	16.0 ± 3.4 a	0.00 ± 0.00	−
	MMY6-2	16.2 ± 1.3 a	0.0 ± 0.0	16.2 ± 1.3 a	0.00 ± 0.00	−
	MMY6-5	13.0 ± 1.5 b	0.0 ± 0.0	13.0 ± 1.5 b	0.00 ± 0.00	−
	MMY3-5	8.1 ± 1.6 c	0.0 ± 0.0	8.1 ± 1.6 c	0.00 ± 0.00	−
	MMY3-7	8.2 ± 1.3 c	0.0 ± 0.0	8.2 ± 1.3 c	0.00 ± 0.00	−

For each cultivar, mean value ± SD in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). Total nodule numbers means the sum of effective and ineffective nodules. The number indicating in table is the mean of 12 plants for all cultivars. + or − show the plants have or do not have the restriction ability.

Table 5. Detection for nodulation incompatibility of *Bradyrhizobium* strains on C244 ($Rj_2Rj_3Rj_4$) soybean variety.

Variety	Isolate	Effective nodule (No. plant^{-1})	Ineffective nodule (No. plant^{-1})	Total nodule (No. plant^{-1})	I/T	Incompatibility
C244	Is-34	2.1 ± 0.3 c	12.8 ± 2.0 a	14.9 ± 2.2 a	0.86 ± 0.02 b	+
($Rj_2Rj_3Rj_4$)	MMY6-1	0.8 ± 0.4 d	11.5 ± 2.5 a	12.3 ± 2.6 *ab*	0.93 ± 0.03 a	+
	MMY6-2	0.7 ± 0.5 d	11.3 ± 2.2 *ab*	11.9 ± 2.3 *ab*	0.95 ± 0.04 a	+
	MMY6-5	2.1 ± 0.6 c	8.2 ± 4.7 b	10.3 ± 5.2 bc	0.76 ± 0.09 c	+
	MMY3-5	10.6 ± 2.2 a	0.0 ± 0.0 c	10.6 ± 2.2 bc	0.00 ± 0.00 d	−
	MMY3-7	8.2 ± 1.3 b	0.0 ± 0.0 c	8.2 ± 1.3 c	0.00 ± 0.00 d	−

For each cultivar, mean value ± SD in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). Total nodule numbers means the sum of effective and ineffective nodules. The number indicating in table is the mean of 12 plants for all cultivars. + or − show the plants have or do not have the restriction ability.

significantly lower than in plants inoculated with MMY3-5 and MMY3-7. The *I/T* ratios of MMY6-1, MMY6-2, MMY6-5, and Is-34 were significantly higher than those of MMY3-5 and MMY3-7. MMY6-1 and MMY6-2 were least compatible with C244, as indicated by their significantly higher *I/T* ratios.

All of the tested strains (Is-34, MMY6-1, MMY6-2, MMY6-5, MMY3-5, and MMY3-7) were highly incompatible with Hill (Rj_4) (*I/T* ratios were in the range 0.86–0.95). MMY3-5 and MMY3-7 were competent formers of effective nodules when associated with Yezin-7 (Rj_4) (Table 6). However, MMY6-1, MMY6-2, MMY6-5, and Is-34 were not compatible with Yezin-7. Hence the *I/T* ratios of MMY6-1, MMY6-2, MMY6-5, and Is-34 associated with Yezin-7 were significantly higher than those of MMY3-5 and MMY3-7. MMY3-5 and MMY3-7 were compatible nodule formers when associated with Fukuyutaka (Rj_4) (Table 6). MMY6-1, MMY6-2, MMY6-5, and *B. japonicum* Is-34 were not competent nodule formers when associated with Fukuyutaka (Rj_4). They formed few effective nodules and large numbers of ineffective nodules. The *I/T* ratios of Is-34, MMY6-1, MMY6-2, and MMY6-5 were 0.82, 0.92, 0.91, and 0.76, respectively, when these strains were associated with Fukuyutaka (Rj_4). The *I/T* ratios of MMY6-1 and MMY6-2 were significantly higher than those of other strains associated with Fukuyutaka (Rj_4), but this

Table 6. Detection for nodulation incompatibility of *Bradyrhizobium* strains on Rj_4 soybean varieties.

Variety (*Rj* gene)	Isolate	Effective nodule (No. plant^{-1})	Ineffective nodule (No. plant^{-1})	Total nodule (No. plant^{-1})	*I/T*	Incompatibility
Hill	Is-34	1.0 ± 0.8 a	5.7 ± 2.5 a	6.7 ± 3.1 a	0.86 ± 0.11 b	+
(Rj_4)	MMY6-1	0.6 ± 0.5 *ab*	5.1 ± 0.8 *ab*	5.7 ± 0.9 *ab*	0.90 ± 0.08 *ab*	+
	MMY6-2	0.0 ± 0.0 b	4.8 ± 1.3 *ab*	4.8 ± 1.3 *ab*	1.00 ± 0.00 a	+
	MMY6-5	0.5 ± 0.5 *ab*	3.7 ± 0.8 b	4.2 ± 0.9 b	0.89 ± 0.12 b	+
	MMY3-5	0.4 ± 0.5 *ab*	4.8 ± 1.0 *ab*	5.2 ± 1.1 *ab*	0.92 ± 0.09 *ab*	+
	MMY3-7	0.3 ± 0.6 *ab*	4.9 ± 1.7 *ab*	5.3 ± 2.0 *ab*	0.95 ± 0.09 *ab*	+
Fukuyutaka	Is-34	1.6 ± 0.6 b	7.4 ± 2.7 a	9.0 ± 3.1 a	0.82 ± 0.06 b	+
(Rj_4)	MMY6-1	0.6 ± 0.5 c	6.8 ± 0.7 *ab*	7.4 ± 0.8 *ab*	0.92 ± 0.07 a	+
	MMY6-2	0.7 ± 0.5 c	5.3 ± 1.8 b	6.0 ± 2.2 b	0.91 ± 0.06 a	+
	MMY6-5	1.3 ± 0.4 bc	4.1 ± 1.0 c	5.3 ± 1.2 b	0.76 ± 0.06 b	+
	MMY3-5	6.1 ± 1.0 a	0.0 ± 0.0 d	6.1 ± 1.0 b	0.00 ± 0.00 c	−
	MMY3-7	6.8 ± 0.8 a	0.0 ± 0.0 d	6.8 ± 0.8 b	0.00 ± 0.00 c	−
Yezin-7	Is-34	0.7 ± 0.6 c	5.6 ± 1.4 b	6.3 ± 1.6 c	0.90 ± 0.09 a	+
(Rj_4)	MMY6-1	0.7 ± 0.9 c	8.8 ± 2.8 a	9.4 ± 3.3 *ab*	0.94 ± 0.08 a	+
	MMY6-2	0.8 ± 0.7 c	5.7 ± 1.4 b	6.5 ± 1.3 bc	0.87 ± 0.12 a	+
	MMY6-5	0.5 ± 0.5 c	9.4 ± 3.5 a	9.9 ± 3.5 a	0.95 ± 0.06 a	+
	MMY3-5	9.6 ± 2.4 a	0.0 ± 0.0 c	9.6 ± 2.4 a	0.00 ± 0.00 b	−
	MMY3-7	7.2 ± 0.9 b	0.0 ± 0.0 c	7.2 ± 0.9 abc	0.00 ± 0.00 b	−
Yezin-11	Is-34	1.0 ± 0.7 c	4.1 ± 1.1 c	5.1 ± 1.6 c	0.83 ± 0.12 b	+
(Rj_4)	MMY6-1	0.5 ± 0.5 c	9.7 ± 1.1a	10.2 ± 0.7 a	0.95 ± 0.05 a	+
	MMY6-2	0.3 ± 0.4 c	7.3 ± 2.6 b	7.5 ± 2.8 b	0.97 ± 0.04 a	+
	MMY6-5	0.4 ± 0.5 c	3.2 ± 1.1 c	3.6 ± 1.4 cd	0.91 ± 0.11 *ab*	+
	MMY3-5	2.9 ± 0.6 b	0.0 ± 0.0 d	2.9 ± 0.6 d	0.00 ± 0.00 c	−
	MMY3-7	4.3 ± 1.6 a	0.0 ± 0.0 d	4.3 ± 1.6 cd	0.00 ± 0.00 c	−
Shan Seine	Is-34	5.2 ± 1.5 b	6.9 ± 1.9 a	12.1 ± 1.5 a	0.57 ± 0.13 b	+
(Rj_4)	MMY6-1	2.1 ± 0.8 c	5.6 ± 1.6 *ab*	7.7 ± 1.5 b	0.72 ± 0.11 a	+
	MMY6-2	2.9 ± 0.8 c	4.0 ± 1.3 b	6.9 ± 1.6 b	0.57 ± 0.10 b	+
	MMY6-5	2.3 ± 0.4 c	4.3 ± 2.0 b	6.6 ± 2.2 b	0.63 ± 0.10 *ab*	+
	MMY3-5	7.3 ± 1.3 a	0.0 ± 0.0 c	7.3 ± 1.3 b	0.00 ± 0.00 c	−
	MMY3-7	7.1 ± 1.1 a	0.0 ± 0.0 c	7.1 ± 1.1 b	0.00 ± 0.00 c	−
Hinthada	Is-34	2.3 ± 1.5 b	4.6 ± 2.1 *ab*	6.8 ± 1.3 *ab*	0.67 ± 0.16 b	+
(Rj_4)	MMY6-1	0.9 ± 0.5 c	4.8 ± 1.2 *ab*	5.7 ± 1.0 b	0.83 ± 0.09 a	+
	MMY6-2	0.7 ± 0.5 c	5.4 ± 1.2 a	6.1 ± 1.6 *ab*	0.90 ± 0.07 a	+
	MMY6-5	1.8 ± 0.6 bc	4.0 ± 0.7 b	5.8 ± 1.1 *ab*	0.70 ± 0.07 b	+
	MMY3-5	7.6 ± 1.7 a	0.0 ± 0.0 c	7.6 ± 1.7 a	0.00 ± 0.00 c	−
	MMY3-7	6.3 ± 0.8 a	0.0 ± 0.0 c	6.3 ± 0.8 *ab*	0.00 ± 0.00 c	−

For each cultivar, mean value ± SD in each column followed by the same letters are not significantly different at $P < 0.05$ (Tukey's test). The number indicating in table is the mean of 12 plants for all cultivars. + or − show the plants have or do not have the restriction ability.

was not the case when they were associated with Hill (Rj_4) (Table 6). MMY3-5 and MMY3-7 formed significantly higher numbers of effective nodules than the other strains when associated with Yezin-11 (Rj_4) (Table 6). MMY6-1, MMY6-2, MMY6-5, and Is-34 were incompatible with Yezin-11. Thus, the *I/T* ratios of MMY6-1, MMY6-2, MMY6-5, and Is-34 were significantly higher than those of MMY3-5 and MMY3-7. MMY6-1 and MMY6-2 had the highest *I/T* ratios. MMY3-5 and MMY3-7 formed significantly higher numbers of effective nodules than other strains when associated with Shan Seine (Rj_4) (Table 6). MMY6-1, MMY6-2, MMY6-5, and Is-34 were incompatible with this cultivar. Hence, the *I/T* ratios of MMY6-1, MMY6-2, MMY6-5, and Is-34 were significantly higher than those of MMY3-5 and MMY3-7. MMY6-1 had the highest *I/T* ratio. MMY3-5 and MMY3-7 formed significantly higher numbers of effective nodules than other strains when associated with Hinthada (Rj_4) (Table 6). MMY6-1, MMY6-2, MMY6-5, and Is-34 were incompatible with Yezin-11. The *I/T* ratios of MMY6-1, MMY6-2, MMY6-5, and Is-34 associated with Yezin-11 were significantly higher than those of MMY3-5 and MMY3-7. MMY6-1 and MMY6-2 had the highest *I/T* ratios.

Overall, the significant difference of *I/T* ratio between Is-34 and MMY6-1/MMY6-2 was not detected on Yezin-7 (Rj_4). Among them, nodule formation on Rj_4 soybean by MMY6-1 strain and MMY6-2 strain was more strongly inhibited than that of Is-34.

4. Discussion

Symbiotic interactions between leguminous plants and *Rhizobium* are highly species-specific, as particular species or strains of rhizobia can perform the symbiotic association with only a specific leguminous species or cultivar (Somasegaran and Hoben 1994). This specificity involves molecular recognition of host plants and bacteria, through the exchange of signaling compounds that induce nodule formation and nitrogen fixation (Denarie *et al*. 1992; Perret *et al*. 2000). Depending on the specificity between host plant and inoculated bacteria, the host plant produce effective or ineffective nodules. Effective nodules are generally large and yellow colored nodules with red pigmentation inside the nodules, and have the ability to perform nitrogen fixation. Ineffective nodules are generally small and white colored nodules with no red pigmentation inside the nodules and cannot perform nitrogen fixation (Htwe and Yamakawa 2017).

The *Rj*(s) and *rj*(s) loci in soybean control nodulation traits after inoculation with compatible *Bradyrhizobium* or *Ensifer/*

Sinorhizobium species. Recessive alleles at three loci, *rj*1, *rj*5, and *rj*6, code for non-nodulating phenotypes (Pracht *et al*. 1993; Williams and Lynch 1954). Furthermore, the dominant alleles Rj_2, Rj_3, Rj_4, and *Rfg1* have unique features that restrict soybean nodulation when the plants are associated with *B. japonicum* USDA122, *B. elkanii* USDA33, *B. elkanii* USDA61 and *E. fredii/S. fredii* USDA257, respectively (Caldwell 1966; Caldwell *et al*. 1966; Trese 1995; Vest 1970; Vest and Caldwell 1972; Weiser *et al*. 1990).

We tested *Bradyrhizobium* strains for nodule formation when associated with Rj_4 soybean cultivars, such as Yezin-11 (Rj_4) and Shan Seine (Rj_4), Yezin-6 (non-*Rj*), CNS (Rj_2Rj_3), and D51 (Rj_3). MMY3-5 and MMY3-7 strains produced effective nodules in all soybean cultivars tested, with the exception of Hill (Rj_4). These findings are congruent with our previous study (Htwe *et al*. 2015a) showing that nodule formation by MMY3-5 and MMY3-7 was restricted in Hill (Rj_4) plants. However, MMY3-5 and MMY3-7 were not classified as type C strains in the current study because they did not inhibit functional nodule formation in Rj_4 genotypes other than Hill (Rj_4). In our previous study, we screened the nodulation traits of indigenous *Bradyrhizobium* isolates in Yezin-6 (non-*Rj*), CNS (Rj_2Rj_3), and Hill (Rj_4) cultivars, and found that MMY3-5 and MMY3-7 produced only one nodule among five Hill (Rj_4) test plants. For this reason, we classified MMY3-5 and MMY3-7 as type C strains. However, we did not count the ineffective nodule numbers or calculate the *I/T* ratios. We calculated *I/T* ratios in the present study and found that MMY3-5 and MMY3-7 performed as type A strains that were able to form nodules when associated with diverse Rj_4 and *Rj* genotype soybean cultivars other than Hill (Rj_4). The *I/T* ratios of these incompatible strains were > 0.5 when they were associated with Rj_4 genotype soybeans. *I/T* ratios > 0.5 indicate incompatibility with specific *Rj* genotype soybean cultivars (Yamakawa *et al*. 1999). There is a possibility that Hill has another unidentified *Rj* genotype that restricts MMY3-5 and MMY3-7 strains in addition to the Rj_4 genotype. Data in Table 6 clearly indicated that genotype of Hill is different from other Rj_4 used here, and this would be a reason of misclassification of strains MMY3-5 and MMY3-7 in our previous work. To identify of nodulation types in *Bradyrhizobium* strain, it is better to test on more than one *Rj* genotype cultivar to acquire the robust results in identifying nodulation types and not to lead the misclassification of nodulation types.

In our studies, we found that MMY6-1, MMY6-2, MMY6-5, and Is-34 were highly compatible with Yezin-6 (non-*Rj*) and CNS (Rj_2Rj_3), but not with Rj_4-genotype soybean cultivars. These findings are in agreement with other reports showing that specific *Bradyrhizobium* strains were unable to form effective nodules on *Rj* genotype soybean cultivars, such as Rj_2, Rj_3, Rj_4, and *Rfg*1 (Caldwell 1966; Caldwell *et al*. 1966; Vest 1970; Vest and Caldwell 1972; Trese 1995; Htwe and Yamakawa 2017). The *I/T* ratios of these incompatible strains were > 0.5. The *I/T* ratios of MMY6-1 and MMY6-2 were significantly higher in some of the Rj_4 soybean cultivars than the *I/T* ratio of the control strain Is-34, i.e., large numbers of ineffective nodules were formed by the strains with high *I/T* ratios. Ineffective nodule is indicated by the presence of numerous small cortical proliferations or protuberances on the roots, indicating that nodulation was initiated but aborted at an early stage. Soybean plants with ineffective nodulation are stunted and appear morphologically similar to nitrogen-deficient control plants that have not been inoculated with bacterial symbionts (Devine and Kuykendall 1996).

The Rj_4 phenotype occurs in 29% of all soybean introductions, and is most frequent in populations from South East Asia; it occurred in > 60% of 800 plants from 12 Asian countries (Devine and Breithaupt 1981). The high frequency of the Rj_4 phenotype in the wild progenitor species and domesticated soybeans in South East Asia suggests that there exists a selection pressure in favor of the Rj_4 phenotype, which is maintained as the predominant form in these populations (Devine and Kuykendall 1996). The occurrence of strongly inhibited strains, such as MMY6-1 and MMY6-2, provides a very useful tool for identifying Rj_4 genotypes in soybean cultivars.

5. Conclusion

The MMY6-1, MMY6-2, MMY6-5, and Is-34 strains formed many ineffective nodules and very few effective nodules when associated with Rj_4 genotype soybean cultivars, but formed many effective nodules in non-*Rj*, Rj_2Rj_3 soybean cultivars. Interestingly, the *I/T* ratios of MMY6-1 and MMY6-2 associated with Rj_4 genotype soybean cultivars, such as C224, Fukuyutaka, Yezin-11, and Hinthada, were significantly higher than that of Is-34. MMY6-1 and MMY6-2 could be used in future studies for the identification of Rj_4 genotypes in soybean cultivars.

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