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Original Paper

Isolation and characterization of rhizobia from nodules of *Clitoria ternatea* in Thailand

Mallika Duangkhet[1], Yamikani Chikoti[1], Apiraya Thepsukhon[2], Pilunthana Thapanapongworakul[3], Sirinapa Chungopast[4], Shigeyuki Tajima[1], Mika Nomura[1,*]

[1] Faculty of Agriculture, Kagawa University, Miki-cho, Kita-gun, Kagawa 761-0795, Japan; [2] Faculty of Agricultural Production, Maejo University, Chiang Mai, 50290, Thailand; [3] Faculty of Agriculture, Chiang Mai University, Huaykaew Rd., Muang,Chiang Mai, 50200, Thailand; [4] Faculty of Agriculture Kamphaeng-saen, Kasetsart University, Nakorn Pathom 73140, Thailand
* E-mail: nomura@ag.kagawa-u.ac.jp Tel: +81-87-891-3135 Fax: +81-87-891-3021

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Abstract Rhizobia were isolated from the root nodules of *Clitoria ternatea* in Thailand. The phylogeny of the isolates was investigated using 16S rDNA and the internal transcribed spacer (ITS) region from 16S to 23S rDNA. The phylogenetic tree of the 16S rDNA showed that ten of the eleven isolates belonged to *Bradyrhizobium elkanii*, and one belonged to *Bradyrhizobium japonicum*. The topology of the ITS tree was similar to that of 16S rDNA. The acetylene reduction activity was higher for the nodules inoculated with the isolated *B. elkanii* strains than for those inoculated with *B. japonicum* strains. When *C. ternatea* plants were inoculated with various *Bradyrhizobium* USDA strains isolated from *Glycine max*, *C. ternatea* formed many effective nodules with *B. elkanii*, especially USDA61. However, acetylene reduction activity per plant and the growth were higher in *C. ternatea* inoculated with our isolates. From these data we propose that effective rhizobia inoculant were identified for *C. ternatea* cultivation.

Key words: 16S rDNA, *Bradyrhizobium*, *Clitoria ternatea*, ITS, nodule.

Introduction

Nodulation is one of the best-known models of symbiotic association between legumes and rhizobia. Host legumes utilize the fixed ammonia from rhizobia as a nitrogen source, and rhizobia are provided with a carbon source. Hence, root nodule symbiosis is mutually beneficial and is of great importance in agriculture as well as in the nitrogen cycle. The formation of root nodules begins with infection inversion through the infection thread by rhizobia. This infection process can be achieved by bacterial signal molecules called Nod factors (Doyle 1994; Lerouge et al. 1990; Schultze and Kondorosi 1998). Nod factors (NFs) are recognized by the Nod factor receptors (NFRs) of the host plant and lead to rhizobia infection through the root hairs. Therefore, the structures of NFs and NFRs are important for host specificity. However, some reports indicate that nodulation formation proceeds without NFs. One is the type 3 secretion system (T3SS) in rhizobia. T3SS is necessary for the virulence of many animal and plant pathogenic bacteria. However, T3SS affects nodulation either positively or negatively, depending on the host plant. The T3SS of *B. elkanii* USDA61 plays a role in promoting nitrogen fixation in soybean *nfr* (Nod factor receptor) mutants but is incompatible with soybean varieties containing the *Rj4* allele (Miwa and Okazaki 2017). The mechanism of the third strategy is not well understood, but it does not involve NFs or the T3SS. This NF-independent and T3SS-independent nodulation occurs in *Aeschynomene evenia* (Fabre et al. 2015).

Rhizobia are soil bacteria that are capable of forming a nitrogen-fixing symbiosis with leguminous plants. Currently, there are 238 recognized species of nodule-forming bacteria on legumes (Shamseldin et al. 2017). However, these identified rhizobia species comprise only approximately 23% of legumes because it has been estimated that there are approximately 19,000 legume species. *Clitoria ternatea* is a member of the Fabaceae family and is generally known as the blue pea or butterfly pea; *C. ternatea* is a local breed in tropical equatorial Asia. Recently, *C. ternatea* was shown to have

Abbreviations: ARA, acetylene reduction activityg; wpi, weeks post infection.

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economic importance related to drug development because of its alternative production of cyclotides, which have anti-HIV or uterotonic activity (Poth et al. 2011). However, there are only few reports that have identified symbiotic rhizobia from *C. ternatea* (Aeron et al. 2015; López-López et al. 2012). Aeron et al. (2015) isolated endophytes that did not produce nodules. López-López et al. (2012) isolated *Rhizobium grahamii* from *C. ternatea* in Mexico. In this study, we isolated a *Bradyrhizobium* strain that can produce effective nodules on *C. ternatea*. Moreover, inoculation tests with various *Bradyrhizobium* USDA strains were performed in *C. ternatea*.

Materials and methods

Strains used in this study

Rhizobia was isolated from *C. ternatea* nodules grown in Chiang Mai, Thailand. Dried nodules were washed with sterile deionized water (SDW) and hydrated with SDW overnight. Surface sterilization of the nodules was carried out by placing them in 70% ethanol for 30 s and then washing them with SDW 5 times. The nodules were then treated with 3% sodium hypochlorite for 1 min, followed by 10 washes with SDW. Each nodule was crushed in 100 μl of 15% glycerol, and 10 μl of the mixture was spread with a loop onto HM medium (Cole and Elkan 1973) containing 1.5% agar and incubated at 28°C for one to two weeks. After two weeks, each colony was incubated on fresh HM agar medium.

Plant growth conditions and inoculation tests

Clitoria ternatea seeds were surface-sterilized and germinated on sterile vermiculite with 0.5× B&D medium (Broughton and Dilworth 1971) in double Magenta jars; the seeds were then inoculated with 10 ml (1×10^9 cells ml^{-1}) of isolates or various soybean *Bradyrhizobium* sp. 5 days later. Plants were grown in a growth cabinet (EYELA FLI-2000; Tokyo Rikakikai Co., Ltd., Japan) at 24°C and exposed to 16 h of light and 8 h of darkness (200 μmol m^{-2} s^{-1}). All strains used in this study are listed in Table 1.

Acetylene reduction activity

After plants were grown for eight weeks post infection (wpi), acetylene reduction activity (ARA) was measured. Ethylene was used as a standard. To measure the ARA, nodules were detached from the roots, placed into a 25 ml vial and incubated at 37°C with 2.6 ml of acetylene. After 30 min, ethylene formation was measured using a Shimadzu GC-8A gas chromatograph (Shimadzu, Kyoto, Japan) (Banba et al. 2001).

Sequence analysis

After single colony isolation, colony PCR was performed. The 16S rDNA and ITS between the 16S and 23S rDNA were amplified with primers as described previously (Itakura et al. 2009). Mighty Amp DNA polymerase (Takara, Osaka, Japan) was used for PCR amplification. The reaction mixture was first incubated at 98°C for 2 min and then subjected to 25 cycles of 98°C for 10 s, 55°C for 15 s and 68°C for 2 min. Amplified DNA fragments were separated on a 1% agarose gel and purified

Table 1. Bacterial strains used in this study.

Strain	Accession no. of DNA sequence		Host plant of source	References
	16S rRNA gene	ITS		
Bradyrhizobium elkanii				
06-1	LC369726	LC369720	*C. ternatea*	This study
09-1	LC369727	LC369721	*C. ternatea*	This study
11-2	LC369728	LC369722	*C. ternatea*	This study
F04-1	LC369730	LC369724	*C. ternatea*	This study
F06-1	LC369731	LC369725	*C. ternatea*	This study
USDA 61	AB110484	EU834736.1	*G. max*	Saeki et al. (2004)
USDA 76^T	U35000	AB100747	*G. max*	van Berkum and Fuhrmann (2000), Saeki et al. (2004)
USDA 94	AF363512	AB100748	*G. max*	Saeki et al. (2004)
USDA101	AF293373.1	AF293373.1	*G. max*	van Berkum and Fuhrmann (2001)
USDA121	AF293374.1	AF293374.1	*G. max*	van Berkum and Fuhrmann (2001)
USDA130	AF208510.1	AF208510.1	*G. max*	van Berkum and Fuhrmann (2001)
Bradyrhizobium japonicum				
F01-1	LC369729	LC369723	*C. ternatea*	This study
USDA 6^T	D85412	AB100741	*G. max*	Ando and Yokoyama (1999), Saeki et al. (2004)
USDA 38	AB231928	AB100743	*G. max*	Sameshima-Saito et al. (2006), Saeki et al. (2004)
USDA 123	AF208504	AB100752	*G. max*	van Berkum and Fuhrmann (2000), Saeki et al. (2004)
USDA 124	AF208505	AB100753	*G. max*	van Berkum and Fuhrmann (2000), Saeki et al. (2004)
USDA 135	AB070571	AB100758	*G. max*	Sameshima-Saito et al. (2006), Saeki et al. (2004)
Bradyrhizobium diazoefficence				
USDA 110	M55485	AB100751	*G. max*	Young et al. (1991), Saeki et al. (2004)
USDA 122	D85408	AB100749	*G. max*	Ando and Yokoyama (1999), Saeki et al. (2004)
Mesorhizobium loti				
MAFF303099	BA000012.4	BA000012.4	*Lotus japonicus*	Kaneko et al. (2000)

using a GFX PCR DNA and gel band purification kit (GE Healthcare, NJ, USA). Direct sequencing was carried out with an ABI PRISM 3700 DNA sequencer and Big Dye terminator v.3.1 cycle sequencing kit (Applied Biosystems, CA, USA).

Phylogenetic analysis

Nucleotide sequences were aligned using the Bioedit program, and phylogenetic trees were constructed using the neighbor-joining method in MEGA version 7 (Kumar et al. 2016); a bootstrap analysis based on 500 repetitions was performed. Nucleotide sequences obtained from the bacterial isolates in this study have been deposited in the DDBJ database. The accession numbers obtained in this study are listed in Table 1.

Results

Phylogenetic analysis of isolated bacteria from C. ternatea

Clitoria ternatea were grown in the soil of Chiang Mai, Thailand, for three weeks. After isolating the bacteria from the nodules, each isolate was tested to confirm the nodulation in *C. ternatea*. Eleven isolates produced nodules in *C. ternatea*. These isolates were sequenced according to their 16S rDNA and the ITS between their 16S and 23S rDNA to create a phylogenetic tree (Figure 1). As a result, ten isolates were classified as *B. elkanii* strains. Each isolate had more than 98% similarity. Five of the ten isolates were identical to each other; therefore, the independent isolates were named 06-1, F06-1, 11-2, 09-1 and F04-1 (Figure 1A). On the other hand, one isolate was classified as a *B. japonicum* strain and named F01-1. The phylogenetic tree for the 16S rDNA and ITS showed that the topology was similar (Figure 1A, B). According to these data, most isolates were classified as *B. elkanii*, suggesting that *B. elkanii* strains predominate in *C. ternatea* nodules in Chiang Mai, Thailand.

Acetylene reduction activity (ARA) in C. ternatea nodules infected with the isolated bacteria

To determine the nitrogen fixing activity, the isolates were infected with *C. ternatea*. After eight weeks post infection, the nodules were measured for acetylene reduction activity (ARA) using gas chromatography. The nodules infected with *B. elkanii* (06-1, F06-1, 11-2, 09-1, and F04-1) showed higher acetylene reduction activity (ARA) compared with those infected with *B. japonicum* strain F01-1 (Figure 2A, B). Consistent with the ARA data, the isolates classified as *B. elkanii* produced many nodules, and their diameters were larger than those of the nodules infected with *B. japonicum* F01-1 (Figure 2C, D). These data indicate that *C. ternatea* can be inoculated with both *B. elkanii* and *B. japonicum*. However, *C. ternatea* nodules have predominately *B. elkanii* and contribute to higher nitrogen fixing activity.

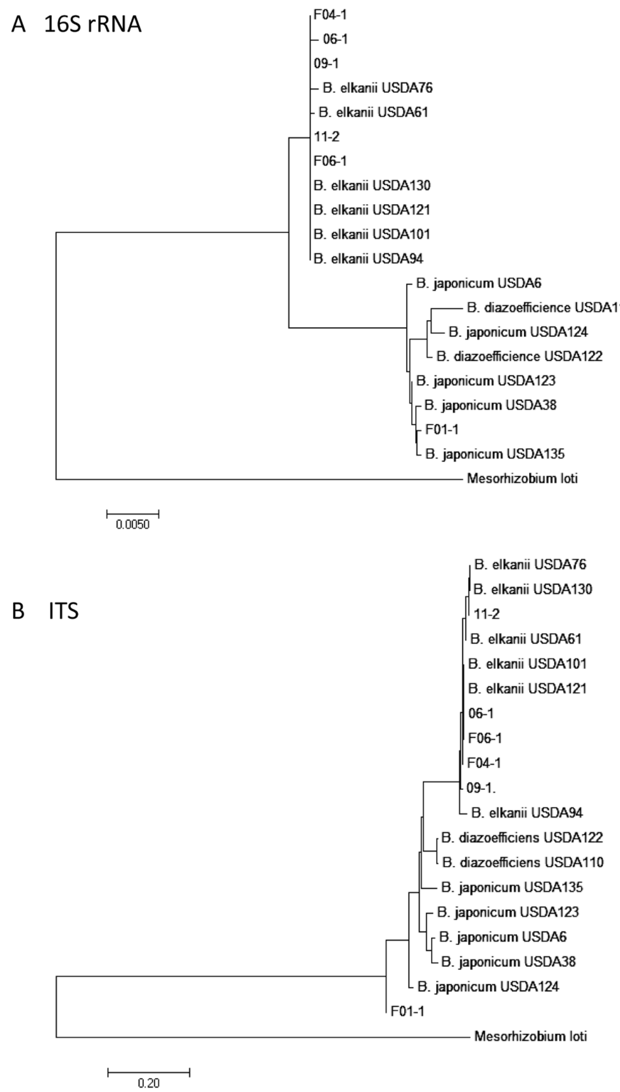

Figure 1. Phylogenetic trees based on the 16S rDNA (A) and ITS region sequences (B) for the genus *Bradyrhizobium* and isolated strains. The branching pattern was produced using the neighbor-joining method. *Mesorhizobium loti* MAFF303099 was used as the outgroup. All accession numbers are listed in Table 1.

Inoculation test with soybean Bradyrhizobium strains in C. ternatea

Glycine max can produce nodules with not only *B. japonicum* but also *B. elkanii*. We next studied whether *C. ternatea* can be inoculated with various soybean-derived rhizobia (Figures 3, 4). Ten strains of *Bradyrhizobium* were used in this study (Table 1). We first expected that *Bradyrhizobium* isolated from soybean would not produce nodules in *C. ternatea* because the Nod factors that activate host signaling are not recognized by the host receptors, NFRs. However, *B. elkanii* strains (USDA61, USDA94, and USDA76) produced nodules with *C. ternatea*. Although *B. diazoefficiens* (USDA110 and 122) was not incompatible with *C. ternatea*, a few nodules were found with *B. japonicum* infection (USDA124 and USDA38) (Figure 3C). Notably, many nodules

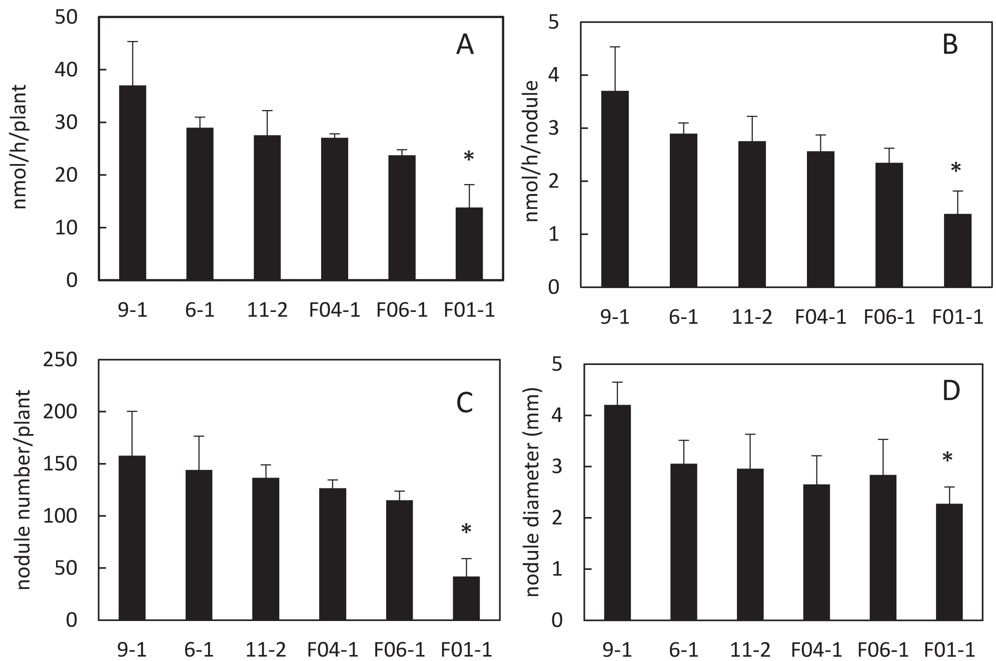

Figure 2. Acetylene reduction activity of *C. ternatea* nodules infected with isolated rhizobia. The plants were grown for eight weeks post infection. Acetylene reduction activity per plant (A), acetylene reduction activity per nodule (B), number of nodules per plant (C), nodule diameter (D). Statistically significant differences compared with 9-1 are indicated by asterisks ($p<0.001$).

were produced with *B. elkanii* USDA61 infection in *C. ternatea* (Figures 3C, 4B). These nodules showed high ARA levels (Figure 3A). When we compared the growth phenotype inoculated with UASA 61 and our isolates, the nodule diameter inoculated with USDA61 was smaller and ARA was lower than that inoculated with isolates from *C. ternatea* (Figures 2, 3). The growth of *C. ternatea* inoculated with USDA61 was slower compared with the plants inoculated with isolates (Figure 4).These data showed that even though *B. elkanii* USDA 61 isolated from soybean can induce significant ARA in *C. ternatea*, isolates from *C. ternatea* in Thailand can induce higher ARA in nodules of *C. ternatea*.

Another phenotype was that although the nodule number was low with *B. elkanii* USDA 76 infection, the nodule diameters and ARA per nodule were more than three times larger than those of the nodules infected with USDA61 (Figure 3). The nodule diameter was significantly larger compared with that infected with isolates from *C. ternatea* (Figures 2D, 3D).

Discussion

Recently, many secondary metabolites, such as triterpenoids, flavonol glycosides anthocyanins and steroids, have been isolated from *C. ternatea* (Mukherjee et al. 2008). Moreover, *C. ternatea* cyclotide, which has uterotonic or anti-HIV activity, is a unique biosynthetic source for cyclotide production (Gilding et al. 2016). Therefore, *C. ternatea* is considered to be economically important because it has been added to various foods and drinks and used as a traditional medicine (Mukherjee et al. 2008). Although *C. ternatea* is an economically important legume plant, there are few reports of *C. ternatea*-rhizobium symbiosis. Rhizobia isolation is beneficial for agriculture because it can utilize biological nitrogen fixation (Wagner 2011). In our study, we isolated rhizobia from *C. ternatea* nodules grown in Chiang Mai, Thailand. The phylogenic tree based on both the 16S rDNA and ITS region sequences showed that two bacteria strains were isolated: one includes *B. elkanii* strains 06-1, F06-1, 11-2, 09-1 and F04-1, and the other is *B. japonicum* F01-1. The sequencing data showed that most of the bacteria isolated from the nodules were *B. elkanii*. Although both *Bradyrhizobium* strains can produce nodules and fix nitrogen in *C. ternatea*, higher acetylene reduction activity levels were found in nodules infected with *B. elkanii* strains (Figure 2). Researchers have previously tried to isolate root nodule bacteria from *C. ternatea* (López-López et al. 2012). They isolated *Rhizobium grahamii* from *C. ternatea* in Mexico. However, we could not isolate the *Rhizobium* strain. These data support that *C. ternatea* nodules predominantly consist of *B. elkanii* strains in Chiang Mai, Thailand.

An inoculation test of soybean-derived *Bradyrhizobium* strains with *C. ternatea* showed that *C. ternatea* infected with *B. elkanii* USDA 61 grew very well compared with the others (Figure 4B). However, ARA from USDA 61 infection was lower than that with our isolated *B. elkanii* strains, indicating that isolated *B. elkanii* strains would be beneficial for *C. ternatea*

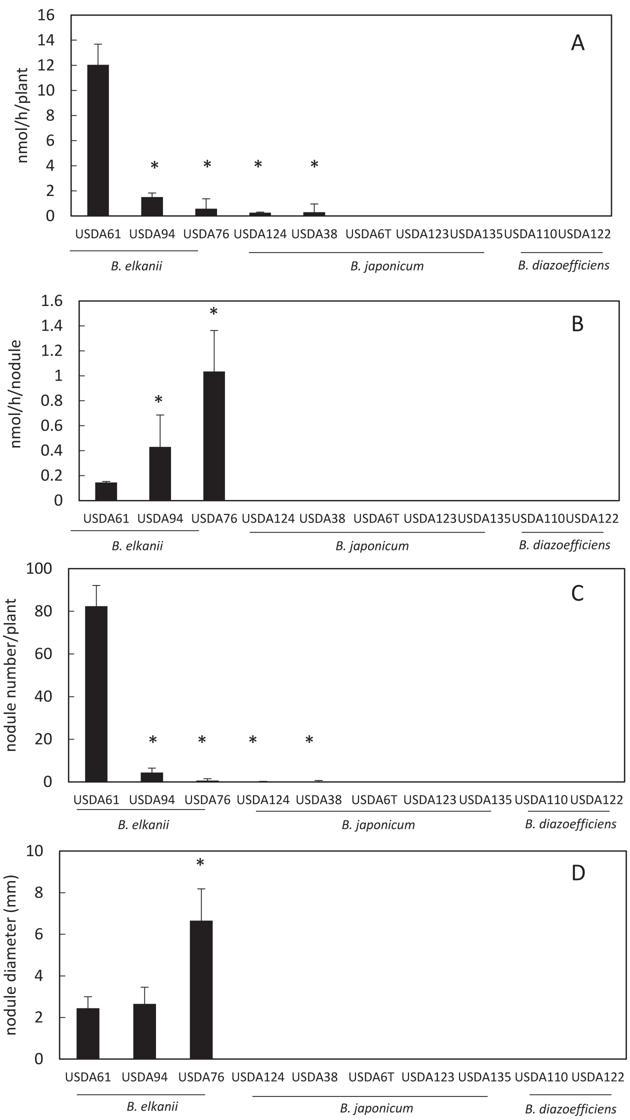

Figure 3. Acetylene reduction activity of *C. ternatea* nodules infected with various *Bradyrhizobium* USDA strains. The plants were grown for eight weeks post infection. Acetylene reduction activity per plant (A), acetylene reduction activity per nodule (B), number of nodules per plant (C), nodule diameter (D). Statistically significant differences compared with USDA61 are indicated by asterisks ($p<0.001$).

cultivation in Thailand.

We do not know why *B. elkanii* USDA 61 can induce nodule in *C. ternatea*. However, Okazaki et al. (2013) showed that *B. elkanii* USDA61 possesses a unique type 3 effector responsible for the activation of nodulation because type 3 secretion system-dependent (T3SS-dependent) nodulation occurs in soybean *nfr1* mutants (Okazaki et al. 2009, 2013). The T3SS would play a role in another infection process through crack entry or intercellular infection without Nod factor signaling. In approximately 25% of legumes found in tropical and warm climates, rhizobia do not invade through infection thread formation (Sprent 2007). Only *B. elkanii* USDA 61 can produce many nodules in *C. ternatea*, suggesting that infection of USDA61 with *C. ternatea* might occur by crack entry or intercellular infection and that type 3 effectors might induce nodulation. However, there is another report that soybeans carrying the *Rj4* allele cannot form nodules with *B. elkanii* USDA61 because of the type 3 effector-triggered immunity (Faruque et al. 2015). Nodulation can be achieved by three strategies: Nod factors, the type 3 secretion system and uncharacterized mechanisms (Masson-Boivin and Sachs 2018). More data are needed to clearly determine the type 3 effector mechanism between *C. ternatea* and *B. elkanii* USDA 61.

It is known that *B. elkanii* can produce rhizobitoxine [2-amino-4-(2-amino-3-hydroxypropoxy) but-3-enoic acid], which is an ethylene synthesis inhibitor, but *B. japonicum* does not (Yuhashi et al. 2000). Ethylene accumulation negatively controls nodulation, and USDA 94 produce high levels of rhizobitoxine (Yuhashi et al. 2000). Minamisawa (1989) reported that *B. elkanii* USDA 76 produces rhizobitoxine. The nodulation caused by USDA 94 and USDA 76 in *C. ternatea* (Figure 3C) might depend on the different accumulation levels of rhizobitoxine. We found *C. ternatea* formed larger nodules with *B. elkanii* USDA 76 even though the number of nodules produced was quite low (Figure 3D). The size was significantly bigger compared with the nodules infected with our isolates (Figures 2D, 3D). The ratio of phytohormones in the nodules might regulate nodule differentiation. Because nodule differentiation and growth are controlled by many types of phytohormones, such as ABA, rhizobia synthesize auxin and cytokinin (Ferguson and Mathesius 2014). Not only phytohormones but also signaling peptides, such as NCR and CLE, control nodule number and cell division (Ferguson and Mathesius 2014; Tatsukami and Ueda 2016). Bacterial infection and accommodation in the nodules depend on rhizobia strain with unknown function (Masson-Boivin and Sachs 2018). Nodule size might be determined by these signaling peptides or phytohormones.

In conclusion, we isolated rhizobia from *C. ternatea* nodules from Chiang Mai Thailand, and most isolates were *B. elkanii*. *Bradyrhizobium elkanii* USDA61 could effectively produce nodules in *C. ternatea*, however, ARA was higher in the nodules infected with isolates from *C. ternatea*. We suggest infection with our isolates, especially *B. elkanii*, is beneficial for high productivity *C. ternatea* cultivation.

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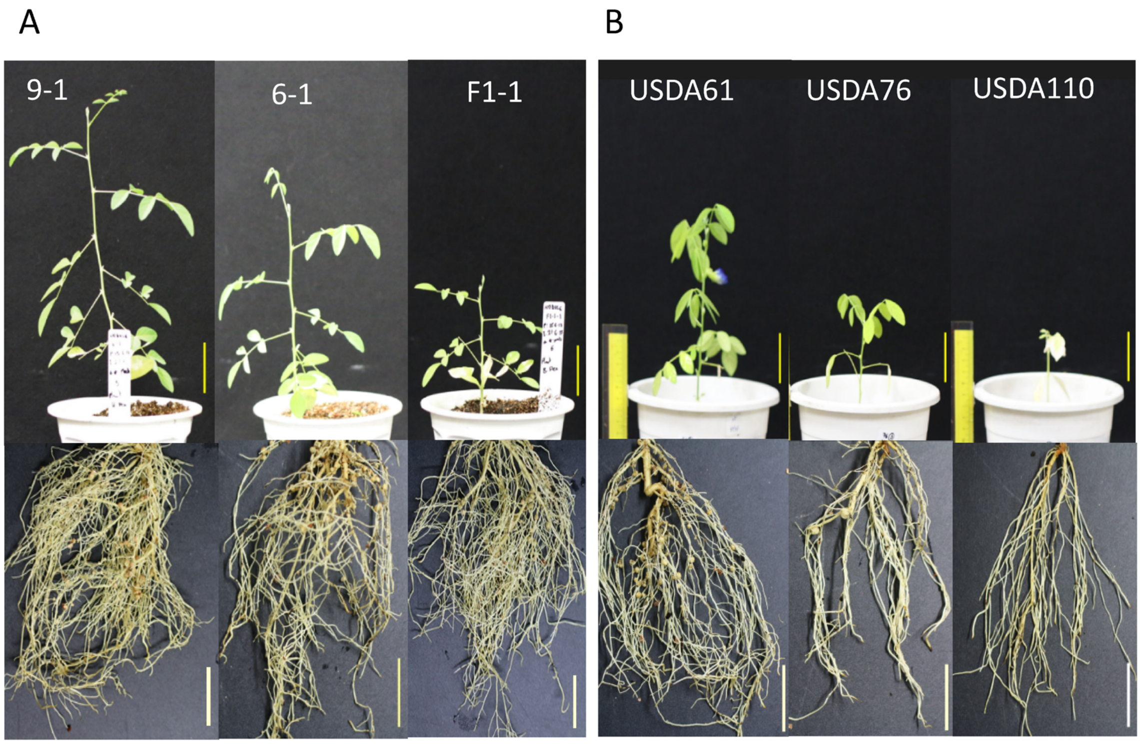

Figure 4. Phenotype of *C. ternatea* infected with various *Bradyrhizobium* strains. The plant shoots and root nodules infected with isolates (A) or *Bradyrhizobium* USDA strains (B) at eight weeks post infection. Bars indicate 9 cm (upper panels), 3 cm (lower panels) respectively.

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