*Journal of Applied and Natural Science*
15(1), 137 - 154 (2023)
ISSN : 0974-9411 (Print), 2231-5209 (Online)
journals.ansfoundation.org

*Research Article*

# Selection and evaluation of *Bradyrhizobium* inoculum for peanut, *Arachis hypogea* production in the Lao People's Democratic Republic

**Tarnee Phimphong**
School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
Faculty of Agriculture, National University of Laos, P. O. Box 7322, Vientiane capital, Lao PDR
**Phoutthasone Sibounnavong**
Faculty of Agriculture, National University of Laos, P. O. Box 7322, Vientiane capital, Lao PDR
**Siviengkhek Phommalath**
Rice and Cash Crop Research Center, National Agriculture Forestry Research Institute (NAFRI), P.O. Box 7170, Vientiane capital, Lao PDR
**Jenjira Wongdee**
School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
**Pongpan Songwattana**
School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
**Pongdet Piromyou**
School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
**Teerana Greetatorn**
School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
**Nantakorn Boonkerd**
School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
**Panlada Tittabutr**
School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand
**Neung Teaumroong***
School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology, Nakhon Ratchasima 30000, Thailand

*Correspondence author. E-mail: neung@sut.ac.th

***Article Info***
https://doi.org/10.31018/jans.v15i1.4270
Received: December 11, 2022
Revised: January 29, 2023
Accepted: February 5, 2023

***How to Cite***
Phimphong, T. *et al.* (2023). Selection and evaluation of *Bradyrhizobium* inoculum for peanut, *Arachis hypogea* production in the Lao People's Democratic Republic. *Journal of Applied and Natural Science*, 15(1), 137 - 154. https://doi.org/10.31018/jans.v15i1.4270

## Abstract
The interaction between leguminous plants and *Bradyrhizobium* is limited, known as host specificity. Therefore, the selection of an appropriate *Bradyrhizobia* for use as biofertilizer inoculum for legumes is necessary. The *Arachis hypogea* L. is the most popular legume produced in the Lao People's Democratic Republic (PDR). Therefore, this research aimed to obtain the appropriate *Bradyrhizobia* that provides high efficiency in *A. hypogea* production in the Lao PDR. The 14 isolates were obtained from root nodules of *A. hypogea* L. trapped with Lao PDR soil samples. Three were the top isolates PMVTL-01, SMVTL-02, and BLXBL-03 showing high efficiency for peanut growth promotion. Strains PMVTL-01 and SMVTL-02 were closely related to the *Bradyrhizobium* geno sp. SA-3 Rp7b and *B. zhanjiangense*, respectively, whilst strain BLXBL-03 was closely related to *Bradyrhizobium* sp. CCBAU51745 and *B. manausense* BR3351. The competitiveness of these strains with *Bradyrhizobium* sp. SUTN9-2::GFP was analyzed, and only *Bradyrhizobium* sp. SMVTL-02 performed a number of occupied nodules higher than SUT-

N9-2::GFP. In addition, the competitiveness of the selected strain *Bradyrhizobium* sp. SMVTL-02 in a soil sample from the Lao PDR in the pot level was employed by tagging the SMVTL-02 with the *DsRed* gene. The results demonstrated that the DsRed-expressing tagged strain showed higher nodule occupancy than indigenous strains. Moreover, the results of the acetylene reduction assay (ARA), nodule number, nodule dry weight, and total plant dry weight from the pot experiment that inoculated with the SMVTL-02::DsRed were presented as having high potential to promote peanut growth as compared to non-inoculation. Thus, *Bradyrhizobium* sp. SMVTL-02 could be considered a potential biofertilizer inoculum for *A. hypogea* production in the Lao PDR.

**Keywords:** *Arachis hypogea* L., Biofertilizer, *Bradyrhizobium* sp., Isolation, Inoculum, Nitrogen fixation

## INTRODUCTION

Nitrogen (N) is the primary limiting nutrient in most terrestrial ecosystems, and it is a nutrient supplied to most plants and a determinant of plant growth (Liu *et al*., 2020). In addition, nitrogen is a component of proteins, enzymes, chlorophyll, and plant growth regulators (Howarth, 2022). Nitrogen deficiency can cause leaf areas, leaf chlorophyll, and the rate of photosynthesis decreases, resulting in reduced biomass production (Yu *et al*., 2022). *Bradyrhizobium* is gram-negative bacteria, capable of symbiosis with leguminous plants (Avontuur *et al*., 2019). *Bradyrhizobium* induces the development of root nodules in leguminous plants and converts atmospheric di-nitrogen to ammonia using the nitrogen-fixing enzyme called nitrogenase (Priyadarshini *et al*., 2021). In most cases, native nitrogen fixers can compete with the inoculum but there are no effective strains and it might not be compatible with the host plant (Irisarri *et al*., 2019). Consequently, inherent dependence on nitrogen-fixing agents without prior information on efficacy and compatibility with legumes leads to crop failure.

The peanut (*Arachis hypogea* L.) is one of the most widespread legumes globally and an important food crop containing high-quality edible oils, easily digestible proteins, and carbohydrates (Ojiewo *et al*., 2020; Shiriki *et al*., 2015). Maize and Cash Crop Research Center, Lao PDR reported (unpublished data) that Lao PDR is a largely agrarian society with 80% of the people living in rural areas. The amount of bean production is about 2%, peanuts shared 1.43% of the total crop production area, with that amount being 77% of bean production. Peanut production in the Lao PDR increased in 2011 by about 30,000 ha, with about 70,000 tons of production. The top three peanut producers in the Lao PDR are the Xayaboury, Salavanh, and Champasack provinces. Small-scale farmers, the major legume producers in the Lao PDR, rarely apply fertilizers during legume production. Hence, the crop is dependent largely on fixed nitrogen from native nitrogen fixers. However, peanut production in the Lao PDR did not usually use biofertilizer. The selection of bradyrhizobia for legume production has received little attention in the Lao PDR due to inadequate research or the negligence of researchers and a lack of awareness of its potential in legume production (Atieno *et al*., 2020). Therefore, an appropriate *Bradyrhizobial* strain for peanut growth promotion in the Lao PDR is required. In view of this, the present study aims to obtain the appropriate *Bradyrhizobia* that provides high efficiency in peanut, *A. hypogea* production in the Lao PDR.

## MATERIALS AND METHODS

### Soil sampling

Soil samples were collected from the area of legume production in three provinces in the Lao PDR: Xayaboury and Xaysomboon provinces, and Vientiane capital (Table S1). The soil samples were collected at 20 samples/site with 1 kg/sample at a depth of 20 cm.

### Isolation of *Bradyrhizobia* from soil samples

Peanut (*Arachis hypogaea* L.) seeds (Lao PDR ecotype) were obtained from the Maize and Cash Crop Research Center, Lao PDR. The peanut seeds were sterilized with 95% alcohol for 20 s and 3% sodium hypochlorite for 20 s and washed with sterilized water 5 times. The seeds were soaked in distilled water for 4 hours and germinated on sterilized vermiculite for 4 days. The pots contained mixed sterilized vermiculite and soil sample (1:1). The sterilized vermiculite was soaked in the water for 1 hour, then put in half of the pot prior to being autoclaved twice. The soil sample was put on top of the sterilized vermiculite. Then, the germinated seed was grown in the pot (1 seed/pot) for 30 days under greenhouse conditions.

After 30 days, the peanut roots were washed thoroughly with tap water to remove soil. The big nodules were selected for 5 nodules/plant. The nodules were put in an Eppendorf tube and the nodules were sterilized with 95% ethanol for 10-20 s, then transferred to soak in 3% sodium hypochlorite for 5 min.s and washed with sterilized water 5 times. The surface-sterilized nodules were cut in half using a pair of blunt-tipped forceps on a sterilized petri dish. Alternatively, the nodules were crushed in an Eppendorf tube with a sterilized glass rod. The one loopful of nodule suspension was streaked on a yeast mannitol (YM) agar plate containing congo red (Somasegaran and Hoben, 2012) and incubated at 30° C for 7 days. The non-absorbed congo red colonies were collected.


**Fig. 1.** *Map showing provinces of Lao PDR and sites of the soil sampling*

**DNA extraction**

Each of the *Bradyrhizobial* isolate was inoculated into HEPES-MES (HM) broth (Cole and Elkan, 1973) at 30° C for 7 days in shaking conditions at 200 rpm. The chromosomal DNA extraction was conducted as described by Hartmann and Amarger (1991).

**BOX-PCR fingerprinting**

BOX-PCR was used to differentiate isolates of the *Bradyrhizobia* using BOX-A1R primer (5'-CTACGGCAAGGCGACGCTGACG- 3') (Versalovic *et al*., 1994). The reaction began with denaturation for 1 min. at 95°C/1 cycle, followed by 35 cycles of denaturation (94°C; 30 s), annealing (94°C; 1 min.) and extension (65°C; 8 min.); followed by a final extension (69°C; 10 min.) for 1 cycle. The BOX-PCR products were visualized by 1.5% agarose gel electrophoresis for 45 min.s at 80 V. The components of the PCR reaction are summarized in Table S2.

**Acetylene reduction assay (ARA) for measuring nitrogenase activity**

The peanut seeds were surface sterilized as mentioned before. Leonard's jar were used to grow the plant employing sterilized vermiculite (Somasegaran and Hoben, 2012). Selected *Bradyrhizobial* strains were cultured in YM broth for 5 days and the cell was washed twice with saline solution (0.85% NaCl) and resuspended using 0.85% NaCl to a final optical density (OD) of 1 at 600 nm. One ml of each inoculant was inoculated into root after the plants were grown for 3 days. In addition, the positive controls were inoculated with *Bradyrhizobium* sp. strains TAL173 and SUTN9-2. All treatments were maintained by Buffered Nodulation Medium (BNM) (Ehrhardt *et al*., 1992) and grown under lightroom conditions, at 25ºC with a 16-h-day and 8-h-night cycle for 30 days.

Plants from each replication were taken from Leonard's jar. The adhering vermiculite was removed by gently shaking and washing. Then, the root nodules were put in an 80 ml test tube that was closed with a rubber stopper. After that, ten percentage of air was removed for an equivalent volume, replaced by acetylene gas; and incubated for 1 hour. The product of ethylene gas in each vial was quantified using a gas chromatograph equipped with a flame ionization detector and a capillary column. The chromatograms were used to integrate the areas of the curves of acetylene ($C_2H_2$) and ethylene ($C_2H_4$) to estimate the activity of nitrogenase in the root nodules (David *et al*., 1980).

**Physiological characteristics**

**Acid-Alkaline production**

This assay was conducted by streaking *Bradyrhizobial* strains on YM agar containing an acid/base indicator, Bromothymol Blue (BTB; 0.025 g/l) (Somasegaran and Hoben, 2012). Plates were incubated at 30°C for 7 days to observe the color changes from green to blue or yellow as indicators of alkali or acid production, respectively. The colony-forming was observed every day with the changing of the color medium.

**IAA (indole-3-acetic acid) production assay**

The IAA production from each strain was determined by a colorimetric technique using the Salkowski reagent containing 1.2% $FeCl_3$ in 37% sulfuric acid (Bric *et al*., 1991). *Bradyrhizobial* strains were first grown as starters for 5 days in YM broth and washed. The cell concentration was measured the same as before. Then, 1% of freshly grown *Bradyrhizobial* culture was cultured in a 50 ml tube containing 15 ml HM broth supplemented with 100 mg/l L-Tryptophan, at 30°C for 7 days with a shaking speed of 190 rpm. The cultures were centrifuged at 5,000 rpm for 10 min.s at 4°C, and the supernatant was mixed with the Salkowski reagent (1:2) (Mohite, 2013). The mixtures were incubated for 15 min.s in darkness at 28 ± 2 °C and absorbance was measured at 530 nm. The produced IAA concentrations were estimated using a standard IAA curve.

**Antibiotic resistance profile**

The *Bradyrhizobial* strains were cultured in YM broth for 5 days and the cell was washed twice with saline solution (0.85% NaCl) and resuspended using 0.85% NaCl to a final optical density (OD) of 1 at 600 nm.

Then, 10 µl of cell suspension in 0.85% NaCl was dropped onto YM agar supplemented with various antibiotics (Table S3) as well as dropped onto YM agar without antibiotics as the control. Three repetitions were done for each test. Colony-forming was observed after 30°C for 7 days.

**Siderophore production assay**

Siderophore production from *Bradyrhizobial* isolates was detected by universal chrome azurol sulfonate (CAS) assay. CAS reagents contained 86.24 mg CAS, 0.142 mM ferric chloride ($FeCl_3.6H_2O$) in 10 mM HCl, and 104.14 mg hexadecyltrimethylammonium bromide (HDTMA) (Arora and Verma, 2017). The *Bradyrhizobial* strains were first grown in YMB for 5 days prior to washing and measurement of the cell concentration, as previously mentioned. Then, 1% of the *Bradyrhizobial* culture was cultivated in nutrient broth (2.5 g/L beef extract, 5 g/L peptone) at 30°C for 7 days. Then, the cultures were centrifuged at 5,000 rpm for 10 min.s at 4°C, and the supernatant was mixed with CAS reagents (1:1). The mixtures were incubated for 20 min.s in darkness at 28 ± 2 °C and absorbance was measured at 630 nm. The siderophore produced by *Bradyrhizobial* strains was measured in percent siderophore unit (psu), controlled according to the formula (Payne, 1993):

Siderophore production PSU = [(Ar – As)/Ar] × 100 Eq.1

Ar = absorbance of the reference (CAS solution and non-injected broth)

As = absorbance of the sample (CAS solution and the cell-free supernatant of the sample)

**Biochemical characteristics**

The *Bradyrhizobial* strains were cultured and washed as previously mentioned. The API ZYM test and carbon utilization and nitrogen assimilation tests were used in this study.

**API ZYM test**

Enzyme activity was measured using the APIZYM kit (bioMerieux). The biochemical characteristics were assessed using the API 20NE kits after 7 days; 20 substrates were used, and the enzyme activity was qualitatively examinate based on color development along with the instructions in the manuscript.

**Carbon utilization and nitrogen assimilation**

Determination of the carbon and nitrogen utilization of *Bradyrhizobial* isolates was conducted by cultivation for 5 days in YMB broth, then the cell culture was washed twice with BNM medium diet without succinate. Cell concentrations were measured at optical density (OD) at 600 nm and taken to $OD_{600}$ of 1 in BNM broth. Carbon (C) and nitrogen (N) sources were added at the appropriate concentrations of 5 mM, 20 mM, and 10 mM, respectively (Table S4), into the liquid BNM medium. To determine the utilization of C and N sources, each test was inoculated with 5% (V/V) bacterial inoculum. Bacterial growth was determined based on culture turbidity, with growth indicated as (+) and no growth indicated as (-) compared to non-inoculation for 7 days in aerated and non-oxygenated broth conditions.

**Nodulation test with other legumes**

*Bradyrhizobial* strains were cultured in YM broth as described previously. The seeds were sterilized with various conditions as summarized in Table S5. Then, seeds were washed with and soaked in sterilized water overnight at ambient temperature, except that *Arachis hypogaea* L. and *Glycine max* were soaked in sterilized water for 4 hours. All seeds were germinated on sterilized 0.8% (w/v) agar for 1 to 2 days at 30°C in the dark. The germinated seeds were transferred into a pouch (two plants/pouch) and inoculated with 2 ml of each strain of bacterial inoculum. All plants were maintained with BNM medium and grown under lightroom conditions at 25ºC with a 16-h-day and 8-h-night cycle for 30 days.

**Genotypic characteristics**

The top three strains (PMVTL-01, SMVTL-02, and BLXBL-03) of *Bradyrhizobial* isolates from different geographical origins were selected. The DNA was amplified with primers 16S rRNA, *recA*, *atpD*, *glnII*, and *rpoB* genes, as listed in Table S6. The 16S rRNA was based on the PCR results. All isolates were shown a single fragment amplification which was subsequently used for sequencing of the 16S rRNA gene. The PCR products of housekeeping genes were transferred into the pTG19-T cloning vector (Vivantis Technologies Sdn Bhd) and screened by blue-white colony selection. The nucleotide sequence of those genes was sequenced by ATGC Co., Ltd., South Korea.

The 16S rRNA*, recA*, *atpD*, *glnII*, and *rpoB* sequences were analyzed and constructed the phylogenetic tree using MEGA (Molecular Evolutionary Genetics Analysis) software version 11.0 with a neighbor-joining tree generated under the K2P distance model and statistical support for tree nodes refers to bootstrap values of 1,000 replicates. Moreover, the phylogenetic tree was combined with the sequences of 16S rRNA and housekeeping genes. The sequences were submitted to the GenBank database. The accession numbers were presented beside the strain's name which showed in each of the phylogenetic tree.

**Co-inoculation of the top three wild type strains with SUTN9-2::GFP**

The top three wild type strains and the SUTN9-2::GFP strain (Piromyou *et al*., 2017) were cultured in YMB, as

described previously. The peanut germinated seeds were transferred to grow in Leonard's jar, after plants were grown for 3 days before being inoculated with inoculum. The ratios of SUTN9-2::GFP per each selected strain ($1\times10^6$ CFU/ml) were 0.1:0.9, 0.3:0.7, 0.5:0.5, 0.7:0.3, and 0.9:0.1 (ml:ml) and the plants were grown under lightroom conditions at 25ºC with a 16-h-day and 8-h-night cycle for 30 days. The best strain was evaluated from nodule occupancy, the nodules derived from SUTN9-2::GFP were observed under a stereo fluorescence microscope (Leica TL5000 Ergo Transmitted Light Base from Leica Microsystems (Switzerland) Ltd.).

**Co-inoculation of SMVTL-02::DsRed in soil condition**

*Bradyrhizobium* sp. strain SMVTL-02 was cultured in HM broth medium for 4 days at 30°C. *Escherichia coli* strain HB101 containing pRK2013 helper plasmid (Figurski and Helinski, 1979) and DH5α harboring the pBjGroEL4::DsRed2 (Hayashi *et al*., 2014) were cultured at 37°C in Luria-Bertani (LB) broth medium (Sambrook *et al*., 1989) supplemented with 100 µg/ml kanamycin (Km) for 24 h. The strain SMVTL-02 was tagged with pBjGroEL4::DsRed2 (Okubo *et al*., 2013) by triparental mating. Transconjugants were selected on HM agar plates containing 200 µg/ml streptomycin and 50 µg/ml nalidixic acid (Okubo *et al*., 2013; Piromyou, Greetatorn, *et al*., 2015).

In this experiment, soil was collected from the Faculty of Agriculture of the National University of Lao PDR campus, located at Nabong, 30 km northeast of Vientiane (18.123680°N, 102.791173°E). In this region, the soil is characterized as sandy loam, which has a pH of 5.07 and contains EC 0.115 ms/cm, OM 1.19%, P 45.20 ppm, K 68.3 ppm, Ca 433.3 ppm, and Mg 89.5 ppm. The seeds were grown in plastic pots (20 cm × 18 cm) containing 1 kg of mixed sterilized vermiculite and 3 kg of the soil sample (1:3) for 4 days (2 plants/pot), After that, the plants were inoculated with SMVTL-02::DsRed 1 ml/plant ($OD_{600}$=1, $1 \times 10^9$ cells/ml). Plants were grown under greenhouse conditions and the ARA, plant dry weight, nodule dry weight, and nodule occupancy were analyzed. The nodule occupancy was analyzed from the derived red nodule by observation under a stereo fluorescence microscope as previously described.

**Statistical analyses**

All experimental data were analyzed by Statistical Package for the Social Sciences (SPSS) software and means were compared using turkey $P < 0.05$. Mean values and standard deviation (SD) were used for data presentation in this experiment.

## RESULTS

**Isolation of *Bradyrhizobial* from soil and strain selection**

To obtain *Bradyrhizobia* nodulating peanuts (Lao PDR ecotype), the soil samples collected from 3 provinces in the Lao PDR, i.e., Xayaboury and Xaysomboon provinces, and Vientiane capital were used for rhizobial isolation using peanut as a root trap. The rhizobial strains were determined heterogeneity between species by BOX-PCR. The result showed that 14 different *Bradyrhizobial* strains were obtained from a total of 250 isolates. Seven strains were isolated from Sanphon village (SPXBL-01, SPXBL-02, SPXBL-03, SPXBL-04, SPXBL-05, SPXBL-06, and SPXBL-07), 3 strains were isolated from Buamlao village (BLXBL-01, BLXBL-02, and BLXBL-03), 2 strains were isolated from Parkxarb mai village (PMVTL-01 and PMVTL-02), and 2 strains were isolated from Somsamai village (SMVTL-01 and SMVTL-02) (Fig. S1).

To select the *Bradyrhizobia* that performed with high efficiency in promoting peanut growth, either equivalent to or better than type strains TAL173 and SUTN9-2, an acetylene reduction assay (ARA) was measured from inoculated peanuts (Fig. S2). The highest nitrogenase activity was derived from isolate PMVTL-01. The ARA activity of isolates SPXBL-02, SPXBL-03, SPXBL-06, BLXBL-03, PMVTL-02, and SMVTL-02 were higher than TAL173 but not significantly different when compared to SUTN9-2. While SPXBL-01, SPXBL-04, SPXBL-05, SPXBL-07, BLXBL-01, BLXBL-02, and SMVTL-01 were lower than SUTN9-2, PMVTL-01, SPXBL-02, SPXBL-03, SPXBL-06, BLXBL-03, PMVTL-02, and SMVTL-02, but not significantly different when compared to TAL173 (Fig. S2A).

*Bradyrhizobium* sp. strain SUTN9-2 performed the highest of nodule number. The isolates SPXBL-01, SPXBL-05, SPXBL-06, SPXBL-07, BLXBL-01, PMVTL-01, PMVTL-02, SMVTL-01, and SMVTL-02 performed significantly higher than TAL173 but significantly lower than SUTN9-2. The isolates SPXBL-02, SPXBL-03, SPXBL-04, BLXBL-02, and BLXBL-03 performed significantly lower than SUTN9-2, SPXBL-01, SPXBL-05, SPXBL-06, SPXBL-07, BLXBL-01, PMVTL-01, PMVTL-02, SMVTL-01, and SMVTL-02 but significantly higher than TAL173 (Fig. S2B).

The nodule dry weight was the highest in SUTN9-2, BLXBL-03, and PMVTL-01 inoculation. The isolates SPXBL-01, SPXBL-02, SPXBL-03, SPXBL-04, SPXBL-05, SPXBL-06, SPXBL-07, BLXBL-01, BLXBL-02, and SMVTL-02 were significantly lower than SUTN9-2, BLXBL-03 and PMVTL-01 but significantly higher than TAL173 (Fig. S2C). Therefore, from these results, the *Bradyrhizobium* sp. isolates PMVTL-01, SMVTL-02, and BLXBL-03 were selected for further study.

**Determination of acid-alkaline and IAA production**

Most of the selected strains were in the slow-grower group (7 days) and generated a medium color from green to blue (alkaline) (Fig. S3). The colony forming size of PMVTL-01 was smaller than that of SMVTL-02 and BLXBL-03; the colony was cream-white, dense, and convex (Fig. S3A). The SMVTL-02 showed a bigger size than PMVTL-01 and BLXBL-03; the colony was translucent, convex, and slime (Fig. S3B). The BLXBL-03 showed a medium colony size as compared to PMVTL-01 and SMVTL-02; the colony was cream-white, dense, and convex (Fig. S3C).

The results of IAA produced from selected isolates demonstrated that BLXBL-03 showed a dark pink color as compared to the other strains (Fig. S4A) and displayed a maximum IAA production at 29.164 µg/ml when compared to the 4 other strains (Fig. S4B). The isolate PMVTL-01 produced IAA at 0.695 µg/ml and SMVTL-02 IAA at 1.282 µg/ml, significantly lower than SUTN9-2 at 6.195 µg/ml. However, they were significantly different when compared to TAL173 at 0.443 µg/ml (Fig. S4B).

**Intrinsic antibiotic-resistant profiles**

The results of antibiotic-resistant profiles indicated that PMVTL-01 resisted carbenicillin, cefotaxime, nalidixic acid, and ampicillin but was sensitive to chloramphenicol, gentamicin, tetracycline, and kanamycin. In addition, this strain showed spontaneity to erythromycin, nalidixic acid (50 µg/ml) medium, streptomycin, and spectinomycin (Table 1). SMVTL-02 resisted carbenicillin and nalidixic acid but was sensitive to chloramphenicol, cefotaxime, gentamicin, tetracycline, kanamycin (100 µg/ml), and ampicillin (100 µg/ml). On the other hand, this strain showed spontaneity to erythromycin, streptomycin, spectinomycin, and kanamycin (50 µg/ml). The strain BLXBL-03 resisted carbenicillin, gentamicin, nalidixic acid, and ampicillin but was sensitive to chloramphenicol and tetracycline and spontaneous to erythromycin, cefotaxime, streptomycin, spectinomycin, and kanamycin. Every selected strain resisted carbenicillin and nalidixic acid and was susceptible to chloramphenicol and tetracycline. On the other hand, BLXBL-03 resisted only gentamicin, and only SMVTL-02 was susceptible to ampicillin (Table 1).

**Determination of siderophore production**

The results of siderophore production indicated that different *Bradyrhizobium* sp. strains showed variation (Fig. S5A and S5B). SMVTL-02 was the most proficient siderophore producer when compared to other strains (Fig. S5B). PMVTL-01 was found to be one of the most efficient siderophore producers, with an efficiency significantly higher than that of SUTN9-2 and BLXBL-03. However, PMVTL-01 was not significantly different when compared to TAL173 (Fig. S5B). Meanwhile, BLXBL-03 showed significantly lower than every strain (Fig. S5B).

**APIZYM test analyses and utilization of various carbon and nitrogen sources**

The result of the APIXYM test demonstrated that enzyme activity derived from all strain tests showed positive results in potassium nitrate (reduction of nitrates to nitrogen), D-glucose (fermentation), L-arginine, urea, esculin, and ferric citrate, as well as negative results for

**Table 1.** *Bradyrhizobium* sp. strains and their intrinsic antibiotic profiles

| Antibiotic | Concentration (µg/ml) | Strains | | |
|---|---|---|---|---|
| | | PMVTL-01 | SMVTL-02 | BLXBL-03 |
| Carbenicillin | 500 | R* | R | R |
| Chloramphenicol | 500 | S | S | S |
| Erythromycin | 250 | SP | SP | SP |
| Cefotaxime | 20 | R | S | SP |
| Gentamicin | 50 | S | S | R |
| Tetracycline | 100 | S | S | S |
| Nalidixic acid | 20 | R | R | R |
| | 50 | SP | R | R |
| Streptomycin | 100 | SP | SP | SP |
| | 200 | SP | SP | SP |
| Spectinomycin | 100 | SP | SP | SP |
| | 200 | SP | SP | SP |
| Kanamycin | 50 | S | SP | SP |
| | 100 | S | S | SP |
| Ampicillin | 50 | R | S | R |
| | 100 | R | S | R |

* Resistant (R), Sensitive (S) and Spontaneous (SP)

**Table 2.** Result of hydrolytic enzymes activity assayed using API ZYM test kit

| Active ingredients | Strains | | | | |
|---|---|---|---|---|---|
| | TAL173 | SUTN9-2 | PMVTL-01 | SMVTL-02 | BLXBL-03 |
| Potassium nitrate (reduction of nitrates to nitrites) | -* | - | - | - | - |
| Potassium nitrate (reduction of nitrates to nitrogen) | + | + | + | + | + |
| L-tryptophan | - | - | - | - | - |
| D-glucose (fermentation) | + | + | + | + | + |
| L-arginine | + | + | + | + | + |
| Urea | + | + | + | + | + |
| Esculin | + | + | + | + | + |
| Ferric citrate | - | - | - | - | - |
| Gelatin (bovine origin) | - | - | - | - | - |
| 4-Nitrophenyl-βD-galactopyranoside | - | - | - | - | - |
| **Assimilation** | | | | | |
| D-glucose | + | + | + | + | + |
| L-arabinose | + | + | + | + | + |
| D-mannose | + | + | - | - | - |
| D-mannitol | + | + | - | - | - |
| N-acetyl-glucosamine | - | - | - | - | - |
| D-maltose | - | - | - | - | - |
| Potassium | - | - | - | - | - |
| Gluconate | - | - | - | - | - |
| Capric acid | - | - | - | - | - |
| Adipic acid | - | - | - | - | - |
| Malic acid | - | - | - | - | - |
| Trisodium citrate | + | + | - | - | - |
| Phenylacetic acid | + | + | - | - | - |

* *Bradyrhizobium* sp. can (+) or cannot (-) use the substrate

**Table 3.** Utilization of various carbon and nitrogen sources by *Bradyrhizobium* sp. in broth culture under anaerobic ($-O_2$) and microaerobic aerobic ($+O_2$) conditions

| Sources | Growth ability observed from culture turbidity (Assimilation) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | TAL173 | | SUTN9-2 | | PMVTL-01 | | SMVTL-02 | | BLXBL-03 | |
| | $+O_2$ | $-O_2$ | $+O_2$ | $-O_2$ | $+O_2$ | $-O_2$ | $+O_2$ | $-O_2$ | $+O_2$ | $-O_2$ |
| **Carbon** | | | | | | | | | | |
| Succinate | +* | + | + | + | + | + | + | + | + | + |
| Malate | - | - | + | + | - | - | - | - | - | - |
| Malonate | - | - | - | - | - | - | - | - | - | - |
| Glucose | + | - | + | - | + | - | + | - | + | - |
| Sucrose | - | - | - | - | - | - | - | - | - | - |
| Phenol | - | - | - | - | - | - | - | - | - | - |
| Glutamate | + | - | + | - | + | - | + | - | + | - |
| Glutamine | + | + | + | + | + | + | + | + | + | + |
| Glycine | - | - | + | - | + | - | - | - | - | - |
| Alanine | - | - | + | + | - | - | - | - | + | + |
| Arginine | - | - | - | - | - | - | - | - | - | - |
| Asparagine | + | + | + | + | + | + | + | + | + | + |
| Proline | + | + | + | + | + | + | + | + | + | + |
| Tryptophan | - | - | - | - | - | - | - | - | - | - |
| Lysine | + | - | + | - | + | - | + | - | + | - |
| Sorbitol | + | + | + | + | + | + | + | + | + | + |
| Myoinositol | - | - | - | - | - | - | - | - | - | - |
| Arabinose | + | - | + | - | + | - | + | - | + | - |
| Mannitol | + | - | + | - | - | - | - | - | - | - |
| **Nitrogen** | | | | | | | | | | |
| $NH_4Cl$ | + | - | + | - | + | - | + | - | + | - |
| $KNO_3$ | + | - | + | - | + | - | + | - | + | - |
| Urea | - | - | - | - | - | - | - | - | - | - |
| $NH_4NO_3$ | + | - | - | - | - | - | - | - | + | - |

**Bradyrhizobium* sp. can (+) or cannot (-) use the substrate

**Table 4** The result of *Bradyrhizobium* sp. nodulation in 18 kinds of legume.

| Plant | Strains | | |
|---|---|---|---|
| | PMVTL-01 | SMVTL-02 | BLXBL-03 |
| *Aeschynomene americana* | +* | + | + |
| *A. indica* | - | - | - |
| *A. evenia* | - | - | - |
| *A. afraspera* | + | + | + |
| *Arachis hypogaea* (Thainan 9) | + | + | + |
| *Cajanus cajan* (Linn.) Millsp. | + | - | + |
| *Crotalaria juncea* | + | + | - |
| *Glycine max* (Chiang Mai 60) | - | - | - |
| *Indigofera tinctoria* | + | + | + |
| *Lotus japonicus* | - | - | - |
| *Macroptilium atropurpureum* | + | + | + |
| *Psophocarpus tetragonolobus* | - | - | - |
| *Samanea saman* | - | - | - |
| *Sesbania rostrata* | - | - | - |
| *Vigna radiata* (SUT1) | + | + | - |
| *V. mungo* (U thong 2) | + | + | + |
| *V. subterranean* | + | + | + |
| *V. unguiculata* ssp. *sesquipedalis* | + | + | + |

**Bradyrhizobium* sp. can (+) or cannot (-) nodulate on legume species

potassium nitrate (reduction of nitrates to nitrites), gelatin (bovine origin), and 4-Nitrophenyl-βD-galactopyranoside (Table 2). In the case of assimilation, the strains TAL173 and SUTN9-2 showed positive results in D-glucose, L-arabinose, D-mannose, and D-mannitol, trisodium citrate, and phenylacetic acid. On the other hand, PMVTL-01, SMVTL-02, and BLXBL-03 showed positive results in D-glucose and L-arabinose but they could not assimilate other substrates tested in this experiment (Table 2).

Most strains could grow on both anaerobic ($-O_2$) and microaerobic aerobic ($+O_2$) conditions with succinate, glutamine, asparagine, proline, tryptophan, and sorbitol (Table 3). Furthermore, TAL173 could grow on glucose ($+O_2$), glutamate ($+O_2$), lysine ($+O_2$), arabinose ($+O_2$), mannitol ($+O_2$), $NH_4Cl$, $KNO_3$, and $NH_4NO_3$. Meanwhile, SUTN9-2 could grow on malate, glucose ($+O_2$), glutamate ($+O_2$), glycine ($+O_2$), alanine, lysine ($+O_2$), arabinose ($+O_2$), mannitol ($+O_2$), $NH_4Cl$, and $KNO_3$. PMVTL-01 could be utilized in glucose ($+O_2$), glutamate ($+O_2$), glycine ($+O_2$), lysine ($+O_2$), arabinose ($+O_2$), $NH_4Cl$, and $KNO_3$. SMVTL-02 was grown in glucose ($+O_2$), glutamate ($+O_2$), lysine ($+O_2$), arabinose ($+O_2$), $NH_4Cl$, and $KNO_3$. BLXBL-03 could utilize in glucose ($+O_2$), glutamate ($+O_2$), alanine, arabinose ($+O_2$), $NH_4Cl$, $KNO_3$, and $NH_4NO_3$.

**Nodulation test with other legumes**

To test the host specificity of *Bradyrhizobium* sp. strains PMVTL-01, SMVTL-02, and BLXBL-03, 18 varieties of legumes were used (Table 4). All the isolates were not nodulated on *Aeschynomene indica*, *A. evenia*, *Glycine max* (Chiang Mai 60), *Lotus japonicus*, *Psophocarpus tetragonolobus*, *Samanea saman*, and *Sesbania rostrata*. In addition, only isolate SMVTL-02 could not nodulate *Cajanus cajan* (Linn.) Millsp. and only isolate BLXBL-03 could not nodulate *Crotalaria juncea* and *Vigna radiata* (SUT1).

**Peanut growth promotion and symbiotic properties**

To determine the nodulation efficiency of new isolated strain PMVTL-01, SMVTL-02 and BLXBL-03 comparing with Thai strains (including *Bradyrhizobium* sp. TAL173 and SUTN9-2), peanut Lao PDR ecotype was used for the nodulation test. From the results of ARA, SMVTL-02 performed highest nitrogenase activity (Fig. 2B). PMVTL-01 showed significantly nitrogenase activity higher than BLXBL-03 and TAL173 but was not significantly different when compared to SUTN9-2 (Fig. 2B). BLXBL-03 performed significantly in terms of lower nitrogenase activity than PMVTL-01, SMVTL-02, and SUTN9-2 but higher than TAL173 (Fig. 2B).

For the nodule numbers, the higher numbers were found in SMVTL-02 and SUTN9-2 (Fig. 2C). PMVTL-01 showed a reduction in nodule numbers, while performed nitrogenase activity higher nodules number than BLXBL-03 and TAL173 (Fig. 2C). BLXBL-03 showed a significantly lower nodule number than every strain except TAL173 (Fig. 2C). The nodule dry weight and total plant dry weight showed nitrogenase activity higher and not significantly different in PMVTL-01, SMVTL-02, and SUTN9-2 (Fig. 2D and 2E), while BLXBL-03 showed a significant reduction but in both nodules, dry weight and total plant dry weight were higher than TAL173 and non-inoculated (Fig. 2D and 2E). These results indicated that SMVTL-02 could be a potential biofertilizer inoculum for peanut production. To confirm efficiency of this strain, the competitiveness of

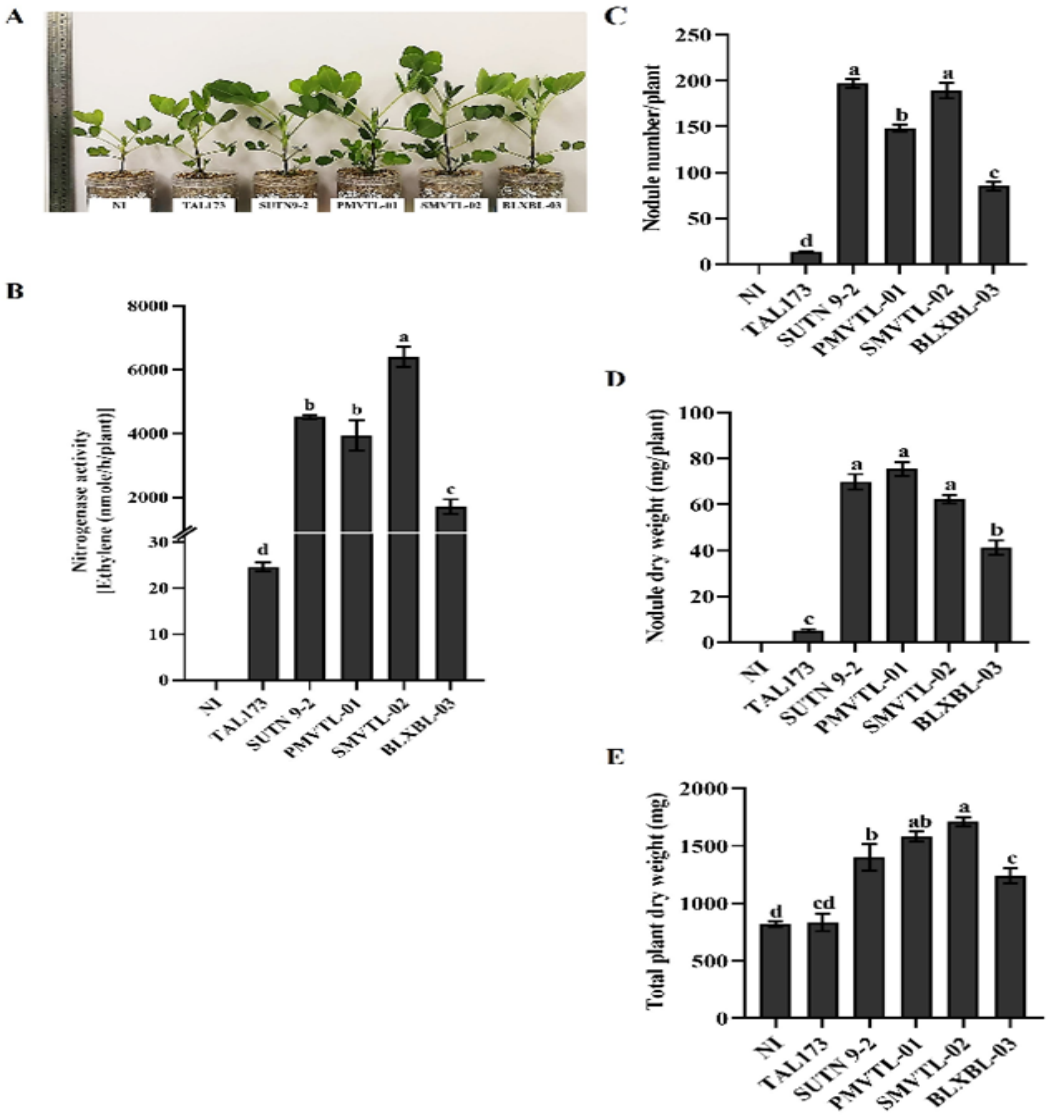


**Fig. 2.** *Peanut growth promotion and symbiotic properties. (A) phenotype of peanut when inoculated with Bradyrhizobium sp., (B) nitrogenase activity measured by acetylene reduction assay (ARA), (C) nodule number, (D) nodule dry weight and (E) total plant dry weight. The results were measured in peanut under lightroom conditions at 30 DAI, non-inoculated (NI). Values with different letters in each treatment differed significantly at P ≥ 0.05 (n = 3)*

SMVTL-02 with indigenous strains was investigated in the next part.

**16S rRNA gene**

To examine the phylogenetic relationships between *Bradyrhizobium* sp. isolates PMVTL-01, SMVTL-02, and BLXBL-03 and other members of the *Bradyrhizobiaceae*, a phylogenetic tree was constructed based on 16S rRNA sequences and *Rhizobium rhizogenes* ATCC11325, *R. tropici* CIAT899, *Sinorhizobium fredii* HH103, and *Mesorhizobium huakuii* USDA4779 were used as an outgroup of the root of the phylogenetic tree (Fig. 3). These results suggested that PMVTL-01 and SMVTL-02 showed the highest similarity to those of *Bradyrhizobium* genosp. SA-3 str. Rp7b and *B. zhanjiangense* CCBAU5178. Furthermore, BLXBL-03 was shown to be closely related to *B. manausense* BR3351 and *B. guangzhouense* with final bootstrap support of 90%.

**Housekeeping gene analysis**

The additional housekeeping genes selected to refine the phylogenetic analysis were conserved among bacteria in the rhizobial group that contains 4 housekeeping. In this study, *recA* (474 bp), *atpD* (512 bp), *glnII* (581bp), and *rpoB* (388 bp) were used for constructing phylogenetic trees (Fig. 4A-D).

In the *recA* gene, PMVTL-01 and SMVTL-02 changed the position closer to *B. liaoningense* LMG18230 and another cluster followed by the first cluster, including

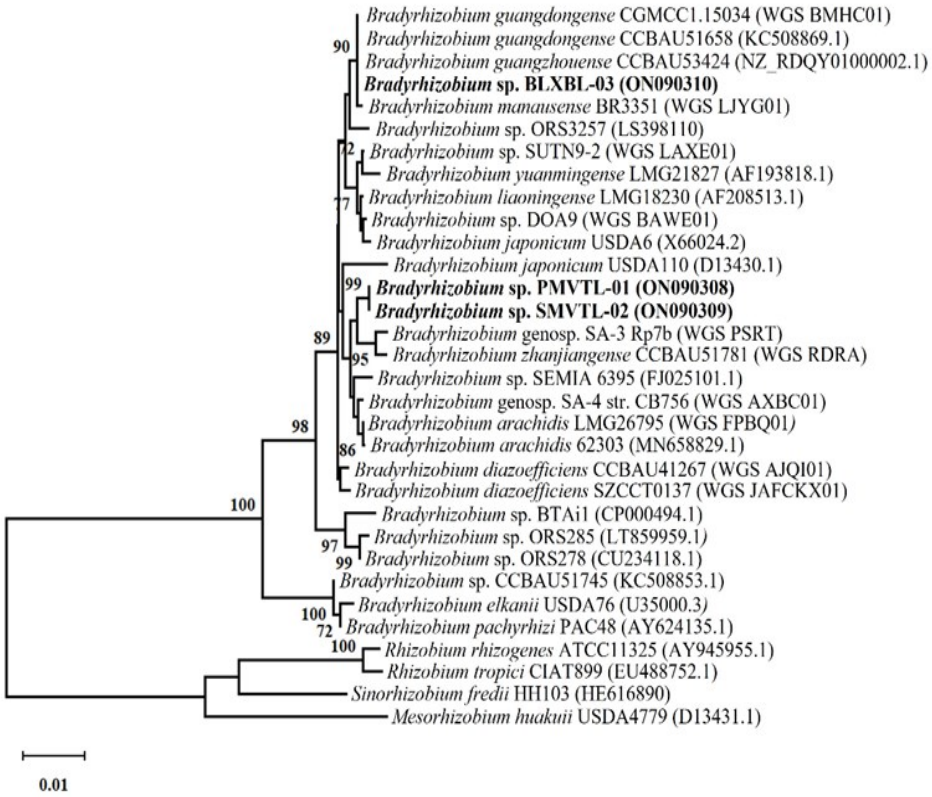


**Fig. 3.** *16S rRNA phylogenetic tree, phylogenetic tree of 16S rRNA sequences acquired by the neighbor-joining method appearing the phylogenetic relationship of the Bradyrhizobium sp. with the correlated species. The phylogenetic tree procreated based on 16S rRNA genes of the 29 rhizobial strains. Neighbor-joining were conducted in MEGA11, the bootstrap is calculated inferred from 1000 replicates and shown at the respective nodes for values >70%, the scale bar represents 0.01% sequence divergence*

*Bradyrhizobium* sp. DOA9, *B. yuanmin.gense* LMG21827, and the second cluster, including *Bradyrhizobium* sp. ORS3257, *Bradyrhizobium* genosp. SA-3 str. Rp7b and *B. zhanjiangense* CCBAU51781. Meanwhile, BLXBL-03 was clustered with *Bradyrhizobium* sp. CCBAU51745 with bootstrap support of 99%, followed by *B. manausense* BR3351 at 51% (Fig. 4A).

The tree built with the *atpD* gene; PMVTL-01 and SMVTL-02 were clustered in *Bradyrhizobium* sp. SUTN9-2 and *B. yuanmin.gense* LMG21827 with bootstrap support of 94%, for BLXBL-03 was closer in two subgroups: the first clustering 4 strains with high similarity with *Bradyrhizobium* sp. CCBAU51745 with bootstrap support of 99% and the second with *B. manausense* BR3351 with bootstrap support of 73% (Fig. 4B).

The tree built with the *glnII* gene also resulted PMVTL-01 and SMVTL-02 being closer in two subgroups: the first clustering 4 strains with higher similarity with *Bradyrhizobium* genosp. SA-4 str. CB756 with bootstrap support of 98% and the second with two strains grouping with *B. arachidis* 62303 and *B. arachidis* LMG26795 with bootstrap support of 82% (Fig. 4C).

A greater variance was also found with the *rpoB* gene; PMVTL-01 and BLXBL-03 showed high similarity to *Bradyrhizobium* sp. ORS3257. Meanwhile, PMVTL-01 was closely relate to *Bradyrhizobium* sp. CCBAU51745 and *B. manausense* BR3351 with bootstrap support of 98% (Fig. 4D).

From the analysis of the 16S rRNA (Fig. 3) and the combination of 4 housekeeping genes (Fig. 5), the section of *Bradyrhizobium* in two main groups was clear and the position of all isolates strains was the same in the 16S rRNA gene tree. PMVTL-01 and SMVTL-02 were closer to *B. zhanjiangense* CCBAU51781 and *Bradyrhizobium* genosp. SA-3 str. Rp7b with bootstrap support of 68%. Meanwhile, BLXBL-03 was relatively homogeneous with two subgroups: the first subgroup including *B. manausense* BR3351 and *Bradyrhizobium sp.* CCBAU51745 with bootstrap support of 99% and the second subgroup including *B. guangdongense* CCBAU51658, *B. guangdongense* CGMCC1.15034, and *B. guangzhouense* CCBAU53424 with bootstrap support of 95%.

**Competitiveness between wild type strain co-inoculated with SUTN9-2::GFP**

To test the competitiveness of wild type strains PMVTL-01, SMVTL-02, and BLXBL-03 compared to *Bradyrhi-*

*zobium* sp. strain SUTN9-2::GFP (Fig. 6A-F), the five-ratio dual-inoculated treatments, strain PMVTL-01 occupied nodules lower than SUTN9-2::GFP, at 1:9 (31.20% SUTN9-2 and 68.80% PMVTL-01), 3:7 (63.03% SUTN9-2 and 36.97% PMVTL-01), 5:5 (57.09% SUTN9-2 and 42.91% PMVTL-01), 7:3 (71.08% SUTN9-2 and 28.92% PMVTL-01), and 9:1 (80.79% SUTN9-2 and 19.21% PMVTL-01) (Fig. 6A and D). The strain SMVTL-02 performed a number of occupied nodules higher than SUTN9-2::GFP, at 1:9 (27.93% SUTN9-2 and 72.07% SMVTL-02), 3:7 (28.84% SUTN9-2 and 71.16% SMVTL-02), 5:5 (38.73% SUTN9-2 and 61.27% SMVTL-02), 7:3 (36.82% SUTN9-2 and 63.18% SMVTL-02), and 9:1

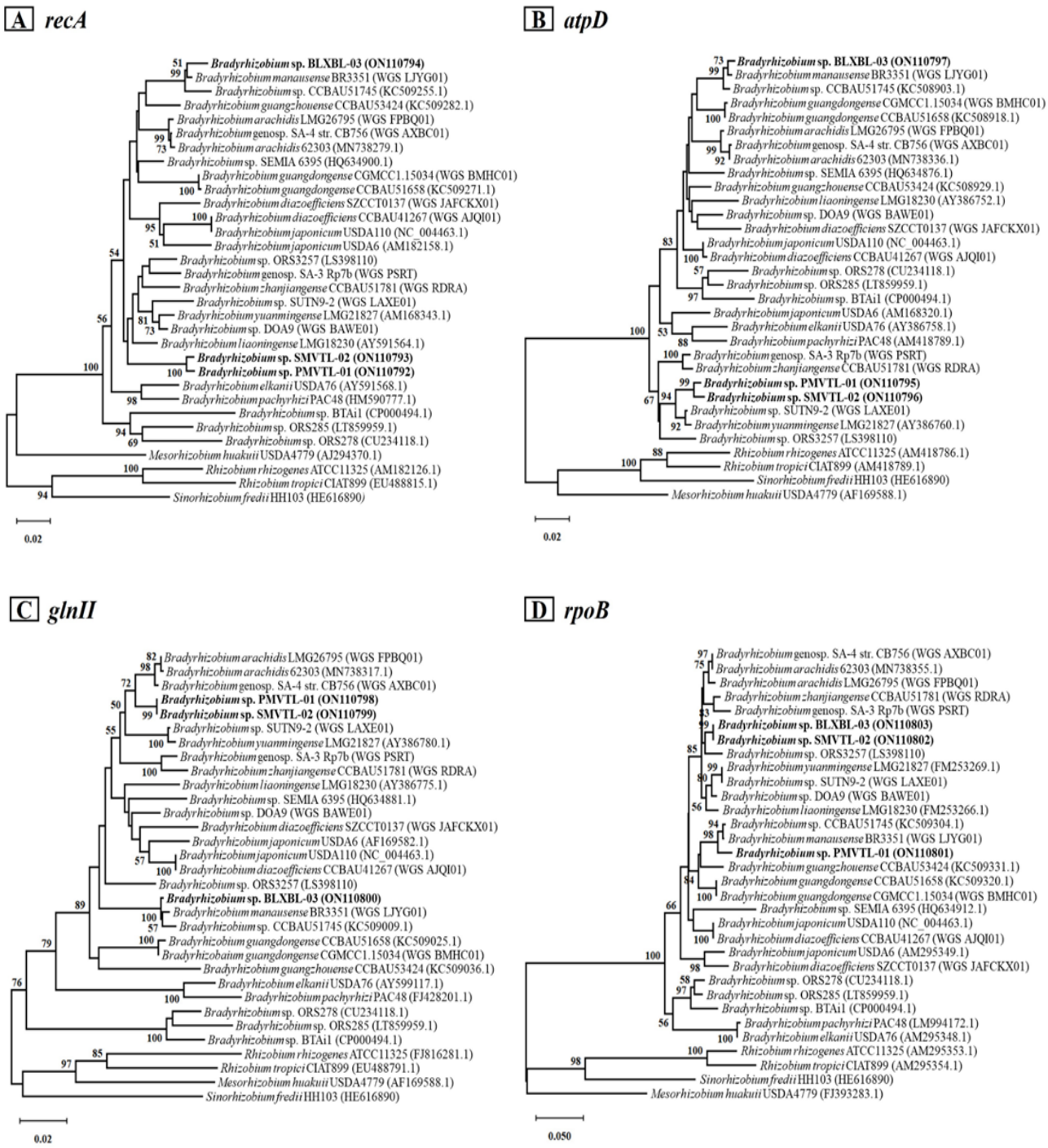


**Fig. 4.** *Phylogenetic tree of recA gene (A), atpD gene (B), glnII gene (C), and rpoB gene (D). The phylogenetic tree procreated based on housekeeping genes were conducted in MEGA11, the bootstrap is calculated inferred from 1000 replicates and shown at the respective nodes for values >50%, the scale bar represents 0.02% sequence divergence*

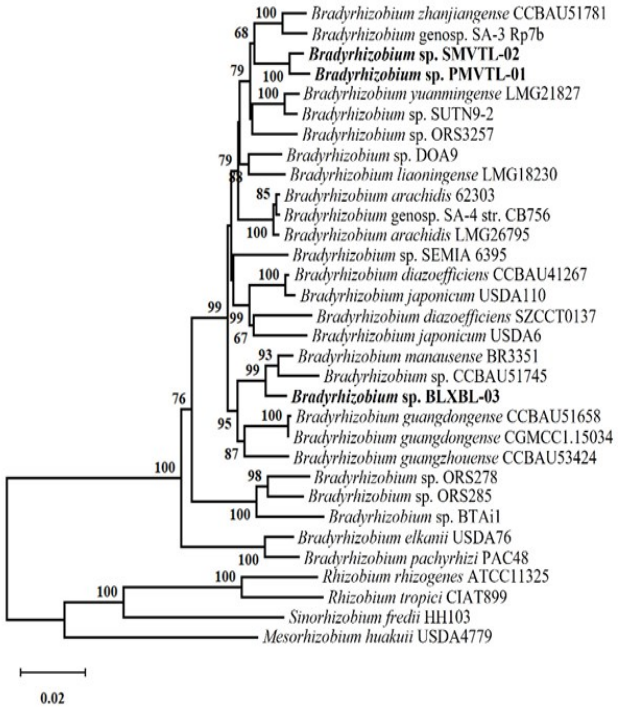


**Fig. 5.** *Sequences of combination five genes (16S rRNA, recA, atpD, glnII and rpoB) were combined in this phylogenetic tree, the phylogenetic tree was conducted in MEGA11, the bootstrap is calculated inferred from 1000 replicates and shown at the respective nodes for values >70%, the scale bar represents 0.02% sequence divergence*

(64.24% SUTN9-2 and 35.76% SMVTL-02) (Fig. 6B and E). In the case of BLXBL-03 showed lower nodule occupancy than SUTN9-2::GFP, the same as PMVTL-01, at 1:9 (36.08% SUTN9-2 and 63.92% BLXBL-03), 3:7 (66.18% SUTN9-2 and 33.82% BLXBL-03), 5:5 (70.71% SUTN9-2 and 29.29% BLXBL-03), 7:3 (66.70% SUTN9-2 and 33.3% BLXBL-03), and 9:1 (78.18% SUTN9-2 and 21.82% BLXBL-03) (Fig. 6C and F). These findings indicate that the competitive ability of strain SMVTL-02 had a higher ability occupancy than SUTN9-2::GFP.

**Competitiveness between SMVTL-02::DsRed and indigenous strains in soil under pot condition**

The determination of the competitiveness of the SMVTL-02::DsRed strain with indigenous strains was carried out on a pot scale under greenhouse conditions. The SMVTL-02::DsRed strain promoted peanut growth such as increased plant height and root length (Fig. 7A and B). The DsRed-expressing tagged strain showed higher nodule occupancy than indigenous strains (81.98% SMVTL-02::DsRed and 18.02% indigenous (Fig. 7C). Moreover, the results of ARA, nodule number, nodule dry weight, and total plant dry weight of the pot inoculated with the SMVTL-02::DsRed strain presented a high potential to promote peanut growth as compared to indigenous strains (Fig. 7C-G).

## DISCUSSION

The results of phylogenetic trees (Fig. 5) indicated that *Bradyrhizobium* sp. isolates PMVTL-01, SMVTL-02 and BLXBL-03 are in the groups of bradyrhizobia containing fewer characterizations and applications in the literature. Therefore, characterizations of strains using a polyphasic approach are necessary prior to application in inoculum production.

**Antibiotic-resistant profiles of isolated strains**

*Bradyrhizobium* sp. isolated from peanuts was shown to be antibiotic-resistant to carbenicillin, nalidixic acid, and spectinomycin but some isolates showed sensitivi-

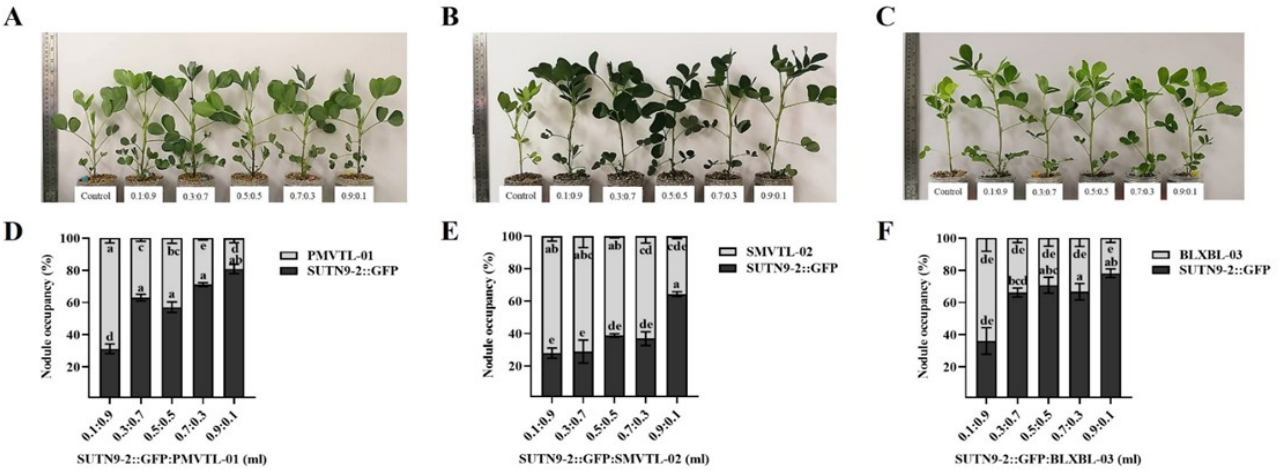


**Fig. 6.** *Competition assay among between Bradyrhizobium sp. strain PMVTL-01, SMVTL-02 and BLXBL-03 and SUTN9-2::GFP strain. (A-C) plant phenotype; (A and D) co-inoculated between PMVTL-01 and SUTN9-2::GFP, (B and E) co-inoculated between SMVTL-02 and SUTN9-2::GFP, and (C and F) co-inoculated between BLXBL-03 and SUTN9-2::GFP. Values with different letters in each treatment differed significantly at P ≥ 0.05 (n = 3).*

ty to spectinomycin (Van Rossum *et al.*, 1995). *B. guangdongense* is resistant to gentamicin but not resistant to chloramphenicol, kanamycin, nalidixic acid, streptomycin, and tetracycline at the minimum dose tested, and *B. guangxiense* is resistant to chloramphenicol, gentamicin, nalidixic acid, streptomycin, and tetracycline but not resistant to kanamycin, trimethoprim, or rifampicin at the minimum dose tested (Li *et al*., 2015). In this study, isolates PMVTL-01, SMVTL-02, and BLXBL-03 are resistant to carbenicillin and nalidixic acid but are not resistant to chloramphenicol, gentamicin, and tetracycline and are spontaneous to erythromycin, streptomycin, and spectinomycin, except that PMVTL-01 is sensitive to kanamycin. When the antibiotic profile of Lao's strains was compared to *B. guangdongense* and *B. guangxiense*, 2 Lao PDR strains (PMVTL-01 and SMVTL-02) were found to be gentamicin sensitive, while both *B. guangdongense* and *B. guangxiense* were resistant.

Intrinsic antibiotic resistance profiles have been used as parameters for rhizobium classification and for the identification of marked strains (Josey *et al*., 1979). Both fast-growing and slow-growing strains of rhizobia showed wide variability in terms of their resistance to antibiotics; fast-growing strains were generally more sensitive than slow-growing strains (Elkan, 1992; Young and Chao, 1989).

**Siderophore production by *Bradyrhizobium sp.***

Bacteria need iron to survive, grow, and develop a variety of mechanisms for the solubility of $Fe^{3+}$ (Guerinot, 1994). Many species of rhizobia, including *Azorhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Rhizobium*, and *Bradyrhizobium*, were reported to produce one or more types of siderophores (Dilworth *et al*., 1998; Johnston, 2004). Tested strains of root nodule bacteria were positive for siderophore production, and the average generation time was unchanged when the iron concentration was increased 200-fold (Carson *et al*., 1992). Our results indicate that SMVTL-02 is the highest siderophore producer, while the lowest is BLXBL-03 (Fig. S5B). Some rhizobacteria insolubly absorb iron from the soil environment and enable plants to use the acid of siderophores; there is evidence that some plants can use bacterial iron (III) from siderophore complexes for plant growth (35–38). On the other hand, the eradication of iron from the soil by rhizobia that produce siderophores reduces iron absorption in the root region and, consequently, inhibits the growth of pathogenic fungi (Bal *et al*., 2013; Traxler *et al*., 2012).

**Biochemical characteristics**

The results of the APIZYM test and the utilization of various carbon and nitrogen sources analyses showed

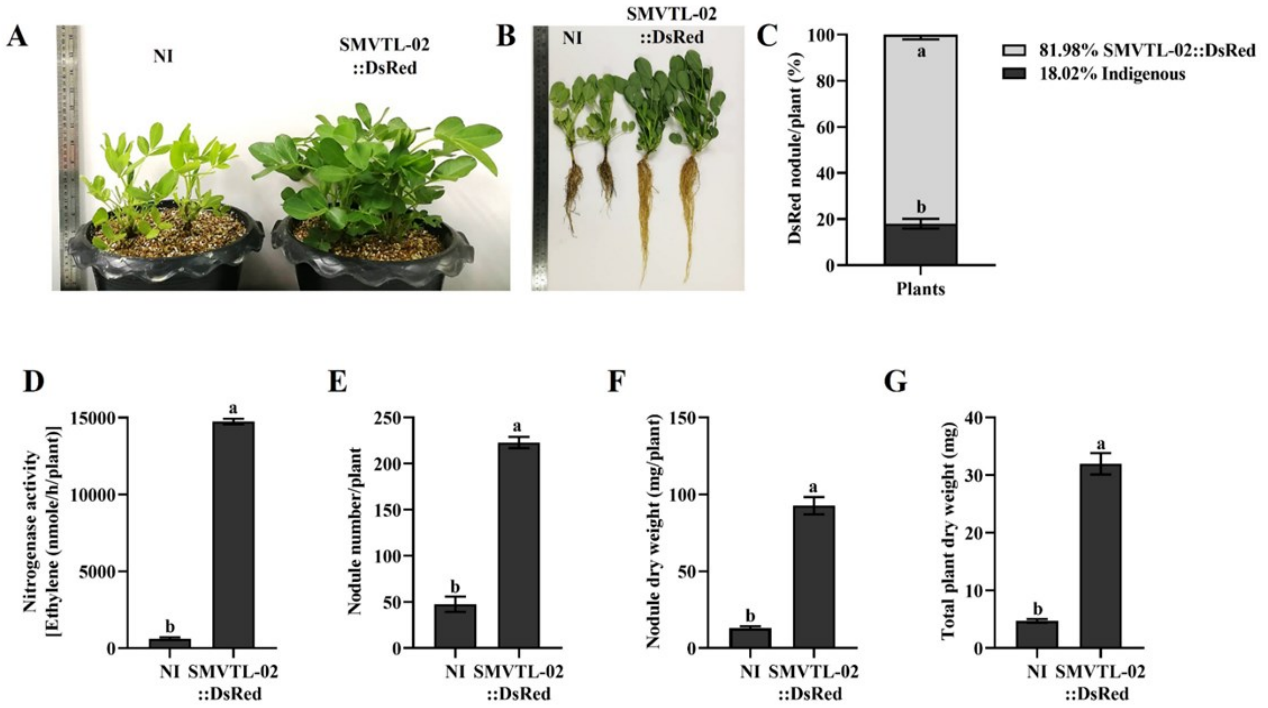


**Fig. 7.** *The competition assay among between Bradyrhizobium sp. strain SMVTL-02::DsRed and indigenous strain. (A-B) plant phenotype, (C) percent of nodules occupancy by SMVTL-02::DsRed and indigenous strains, (D) nitrogenase activity, (F) Nodule dry weight and (G) Total plant dry weight. The results were measured in peanut under lightroom conditions at 30 DAI, non-inoculated (NI). Values with different letters in each treatment differed significantly at P ≥ 0.05 (n = 3)*

a variety of utilization patterns among different strains of *Bradyrhizobium* sp. carbon resource and nitrogen utilization patterns are not strongly related to the symbiovar or genospecies (Kumar *et al*., 2015; Li *et al*., 2019). Some carbon or nitrogen sources varied in the assimilating ability of the species, which indicates the phenotypic diversity within the species (Martins da Costa *et al*., 2019). Most of the *Bradyrhizobium* sp. strains in this study did not utilize disaccharides including sucrose and maltose, which primarily did not have a disaccharide absorption system (Elkan, 1992; Glenn and Dilworth, 1981). However, most of the strains, such as *B. elkanii* USDA 76, *B. liaoningense* USDA 3622, *B. yuanmingense* CCBAU 10071, *B. diazoefficiens* USDA $110^{T}$, and *B. japonicum* USDA 6 were grown on glucose (Wójcik *et al*., 2019).

**Efficiency of nodulation test with other legumes**

The *Bradyrhizobium* sp. could efficiently nodulate many plant hosts (Azarias Guimarães *et al*., 2015; Stacey *et al*., 1992) and there can infecting specific legumes and establishing nitrogen-fixing symbiosis with them (Ferguson, 2017). Many reports revealed that the *Bradyrhizobium* sp. nodulated *Aeschynomene mericana* (Noisangiam *et al*., 2012; Nzoué *et al*., 2009), *A. afraspera* (Bonaldi *et al*., 2011), *Arachis hypogaea* (Thainan 9) (Songwattana *et al*., 2017), *Cajanus. cajan (Linn.) Millsp.* (Alaswad *et al*., 2019), *C. juncea* (You *et al*., 2002), *Indigofera tinctoria* (Leelahawonge *et al*., 2010), *Macroptilium atropurpureum* (Yuhashi *et al*., 2000), *Vigna radiata* (SUT1) (Songwattana *et al*., 2021), *V. mungo* (U thong 2) (Piromyou *et al*., 2021), *V. subterranean* (Puozaa *et al*., 2017), and *V. unguiculata* ssp. *sesquipedalis* (Kathalia *et al*., 2020). However, SMVTL-02 could not nodulate on *C. cajan* and BLXBL-03 could not nodulate on *C. juncea* and *V. radiata* (SUT1).

**Genotypic characteristics of isolates**

The 16S rRNA gene was used worldwide for the classification and identification of bacteria and rhizobia. Recently, the housekeeping genes have proven useful for classification and identifying another group of rhizobia (Gaunt *et al*., 2001; Islam *et al*., 2008; Klepa *et al*., 2022; Ngwenya *et al*., 2022; Rivas *et al*., 2009). The strains PMVTL-01, SMVTL-02, and BLXBL-03 displayed a diversity closely related to many species of *Bradyrhizobium* when housekeeping genes were analyzed. However, their position is quite the same as compared to the tree of 16S rRNA and five combinations of genes. The strains PMVTL-01 and SMVTL-02 were closely related to the *Bradyrhizobium* genosp. SA-3 Rp7b and *B. zhanjiangense*, while strain BLXBL-03 was closely related to *Bradyrhizobium* sp. CCBAU51745, *B. manausense* BR3351, *B. guangdongense* CCBAU51658, *CGMCC*1.15034, and *B. guangzhouense* CCBAU53424. All of the closely related strains in this phylogenetic tree were isolated from *A. hypogaea* (Li *et al*., 2015, 2019), except *B. manausense* BR3351, which was isolated from *V. unguiculata* (Silva *et al*., 2014).

**Competitiveness of isolate strains with SUTN9-2::GFP**

The percent of nodule occupancy of SMVTL-02 was higher than that of SUTN9-2::GFP, which might imply that SUTN9-2 was not originally isolated from *A. hypogaea* but instead was isolated from root nodules of *A. americana* (Noisangiam *et al*., 2012). Factors affecting nodulation competitiveness vary according to the partnership between legumes and rhizobia. This pattern might be due to differences in the bacteria's metabolic capacity, infectious processes, and the life history of bacteria within the nodules (Archana, 2010; Prell and Poole, 2006). From the result, SUTN9-2 produces less siderophore than SMVTL02 (Fig. S5B), which might render less competition. The level of siderophore produced affected nodulation efficiency (Rajendran *et al*., 2008; Sturz *et al*., 1997). Moreover, the exchange of C and N between bacteria and plant cells suggests that the metabolic traits of rhizobia may also be important during the endophytic part of the bacterial life cycle (Prell and Poole, 2006; White *et al*., 2007).

**Competitiveness of SMVTL-02::DsRed with indigenous strains**

The peanut is a highly promiscuous species because it is nodulated by the rhizobia that also nodulate a variety of other legumes (Alwi *et al*., 1989; Bogino *et al*., 2006). The response of peanuts to inoculation with bradyrhizobia is affected by many factors including the effects of indigenous rhizobia populations (Bogino *et al*., 2008). However, some rhizobia are unable to nodulate peanuts to be effective at nitrogen-fixing in symbiosis with plants. Thus, good inoculum strains must be able to survive and adapt to new environments, to outnumber and prevail the rhizobia an indigenous strain (Miljakovic *et al*., 2022; Yates *et al*., 2011). In this experiment, few nodules (18.02%) were formed by the indigenous strains; 81.98% were formed by the SMVTL-02::DsRed strain, indicating that soil has an indigenous rhizobia population that can convince the nodulating on peanut plants, although the nitrogen fixation efficiency is low. Moreover, in treatment inoculated with SMVTL-02::DsRed strain had the greatest enhancing effect on nodulation and plant growth.

## Conclusion

The results of this study showed the high genetic diversity in the group of *Bradyrhizobial* symbionts of peanuts in Lao soil. The top three high-efficiency strains, i.e.,

PMVTL-01, SMVTL-02, and BLXBL-03, were selected from 14 different strains of the nodulating peanut root. All the selected strains belonged to the genus *Bradyrhizobium* based on 16S rRNA, *recA*, *atpD*, *glnII*, and *rpoB* gene sequences. Regarding the determination of competitiveness, SMVTL-02 had the greatest efficiency of nodulation and nitrogen fixation as compared to the SUTN9-2::GFP strain and indigenous strains. Therefore, SMVTL-02 has promising potential for development as a biofertilizer inoculum for peanut production in the Lao PDR.

## ACKNOWLEDGEMENTS

This work was carried out at the School of Biotechnology, Institute of Agriculture Technology, Suranaree University of Technology. The following funding bodies are acknowledged for financial support by (i) the Asian Development Bank-SPS-GMS project, Faculty of Agriculture, National University of Laos [grant number OPN. 4019], (ii) the NSRF via the Program Management Unit for Human Resources & Institutional Development, Research and Innovation [grant number B16F640113].

### Conflict of interest

The authors declare that they have no conflict of interest.

## REFERENCES

1. Alaswad, A. A., Oehrle, N. W. & Krishnan, H. B. (2019). Classical Soybean (*Glycine max* (L.) Merr) Symbionts, *Sinorhizobium fredii* USDA191 and *Bradyrhizobium diazoefficiens* USDA110, Reveal Contrasting Symbiotic Phenotype on Pigeon Pea (Cajanus cajan (L.) Millsp). *International Journal of Molecular Sciences*, *20*(5), Article 5. https://doi.org/10.3390/ijms20051091
2. Alwi, N., Wynne, J. C., Rawlings, J. O., Schneeweis, T. J. & Elkan, G. E. (1989). Symbiotic relationship between *Bradyrhizobium* strains and peanut. Crop Science. *Crop Science*, *29*, 50–54.
3. Archana, G. (2010). Engineering Nodulation Competitiveness of Rhizobial Bioinoculants in Soils. In M. S. Khan, J. Musarrat & A. Zaidi (Eds.), *Microbes for Legume Improvement* (pp. 157–194). Springer. https://doi.org/10.1007/978-3-211-99753-6_8
4. Arora, N. K. & Verma, M. (2017). Modified microplate method for rapid and efficient estimation of siderophore produced by bacteria. *3 Biotech*, *7*(6), 381. https://doi.org/10.1007/s13205-017-1008-y
5. Atieno, M., Herrmann, L., Nguyen, H. T., Phan, H. T., Nguyen, N. K., Srean, P., Than, M. M., Zhiyong, R., Tittabutr, P., Shutsrirung, A., Bräu, L. & Lesueur, D. (2020). Assessment of biofertilizer use for sustainable agriculture in the Great Mekong Region. *Journal of Environmental Management*, *275*, 111300. https://doi.org/10.1016/j.jenvman.2020.111300
6. Avontuur, J. R., Palmer, M., Beukes, C. W., Chan, W. Y., Coetzee, M. P. A., Blom, J., Stępkowski, T., Kyrpides, N. C., Woyke, T., Shapiro, N., Whitman, W. B., Venter, S. N. & Steenkamp, E. T. (2019). Genome-informed *Bradyrhizobium* taxonomy: Where to from here? *Systematic and Applied Microbiology*, *42*(4), 427–439. https://doi.org/10.1016/j.syapm.2019.03.006
7. Azarias Guimarães, A., Florentino, L. A., Alves Almeida, K., Lebbe, L., Barroso Silva, K., Willems, A. & de Souza Moreira, F. M. (2015). High diversity of *Bradyrhizobium* strains isolated from several legume species and land uses in Brazilian tropical ecosystems. *Systematic and Applied Microbiology*, *38*(6), 433–441. https://doi.org/10.1016/j.syapm.2015.06.006
8. Bal, H. B., Das, S., Dangar, T. K. & Adhya, T. K. (2013). ACC deaminase and IAA producing growth promoting bacteria from the rhizosphere soil of tropical rice plants. *Journal of Basic Microbiology*, *53*(12), 972–984. https://doi.org/10.1002/jobm.201200445
9. Bogino, P., Banchio, E., Bonfiglio, C. & Giordano, W. (2008). Competitiveness of a *Bradyrhizobium* sp. Strain in Soils Containing Indigenous Rhizobia. *Current Microbiology*, *56*(1), 66–72. https://doi.org/10.1007/s00284-007-9041-4
10. Bogino, P., Banchio, E., Rinaudi, L., Cerioni, G., Bonfiglio, C. & Giordano, W. (2006). Peanut (*Arachis hypogaea*) response to inoculation with Bradyrhizobium sp. In soils of Argentina. *Annals of Applied Biology*, *148*(3), 207–212. https://doi.org/10.1111/j.1744-7348.2006.00055.x
11. Bonaldi, K., Gargani, D., Prin, Y., Fardoux, J., Gully, D., Nouwen, N., Goormachtig, S. & Giraud, E. (2011). Nodulation of *Aeschynomene afraspera* and A. indica by Photosynthetic *Bradyrhizobium* Sp. Strain ORS285: The Nod-Dependent Versus the Nod-Independent Symbiotic Interaction. *Molecular Plant-Microbe Interactions®*, *24*(11), 1359–1371. https://doi.org/10.1094/MPMI-04-11-0093
12. Bric, J. M., Bostock, R. M. & Silverstone, S. E. (1991). Rapid In Situ Assay for Indoleacetic Acid Production by Bacteria Immobilized on a Nitrocellulose Membrane. *Applied and Environmental Microbiology*, *57*(2), 535–538. https://doi.org/10.1128/aem.57.2.535-538.1991
13. Carson, K. C., Holliday, S., Glenn, A. R. & Dilworth, M. J. (1992). Siderophore and organic acid production in root nodule bacteria. *Archives of Microbiology*, *157*(3), 264–271. https://doi.org/10.1007/BF00245160
14. Cole, M. A. & Elkan, G. H. (1973). Transmissible Resistance to Penicillin G, Neomycin, and Chloramphenicol in *Rhizobium japonicum*. *Antimicrobial Agents and Chemotherapy*, *4*(3), 248–253. https://doi.org/10.1128/AAC.4.3.248
15. David, K. A. V., Apte, S. K., Banerji, A. & Thomas, J. (1980). Acetylene Reduction Assay for Nitrogenase Activity: Gas Chromatographic Determination of Ethylene Per Sample in Less Than One Min.. *Applied and Environmental Microbiology*, *39*(5), 1078–1080. https://doi.org/10.1128/aem.39.5.1078-1080.1980
16. Dilworth, M. J., Carson, K. C., Giles, R. G. F., Byrne, L. T. & Glenn, A. R. Y. 1998. (1998). *Rhizobium leguminosarum* bv. Viciae produces a novel cyclic trihydroxamate siderophore, vicibactin. *Microbiology*, *144*(3), 781–791. https://doi.org/10.1099/00221287-144-3-781
17. Ehrhardt, D. W., Atkinson, E. M. & Long, S. R. (1992). Depolarization of Alfalfa Root Hair Membrane Potential by *Rhizobium meliloti* Nod Factors. *Science*, *256*(5059), 998–

1000. https://doi.org/10.1126/science.10744524
18. Elkan, G. (1992). *Taxonomy of the rhizobia*. https://cdnsciencepub.com/doi/abs/10.1139/m92-075
19. Ferguson, B. J. (2017). Rhizobia and Legume Nodulation Genes□. In *Reference Module in Life Sciences*. Elsevier. https://doi.org/10.1016/B978-0-12-809633-8.07071-0
20. Figurski, D. H. & Helinski, D. R. (1979). *Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans*. https://doi.org/10.1073/pnas.76.4.1648
21. Gaunt, M. W., Turner, S. L., Rigottier-Gois, L., Lloyd-Macgilp, S. A. & Young, J. P. 2001. (2001). Phylogenies of *atpD* and *recA* support the small subunit rRNA-based classification of rhizobia. *International Journal of Systematic and Evolutionary Microbiology*, *51*(6), 2037–2048. https://doi.org/10.1099/00207713-51-6-2037
22. Glenn, A. R. & Dilworth, M. J. (1981). The uptake and hydrolysis of disaccharides by fast-and slow-growing species of *Rhizobium*. *Archives of Microbiology*, *129*(3), 238–239. https://doi.org/10.1007/BF00425257
23. Guerinot, M. L. (1994). Microbial iron transport. *Annual Review of Microbiology*, *48*, 743–773.
24. Hartmann, A. & Amarger, N. (1991). Genotypic diversity of an indigenous *Rhizobium meliloti* field population assessed by plasmid profiles, DNA fingerprinting, and insertion sequence typing. *Canadian Journal of Microbiology*, *37*(8), 600–608. https://doi.org/10.1139/m91-102
25. Hayashi, M., Shiro, S., Kanamori, H., Mori-Hosokawa, S., Sasaki-Yamagata, H., Sayama, T., Nishioka, M., Takahashi, M., Ishimoto, M., Katayose, Y., Kaga, A., Harada, K., Kouchi, H., Saeki, Y. & Umehara, Y. (2014). A Thaumatin-Like Protein, Rj4, Controls Nodule Symbiotic Specificity in Soybean. *Plant and Cell Physiology*, *55*(9), 1679–1689. https://doi.org/10.1093/pcp/pcu099
26. Howarth, R. W. (2022). Nitrogen□. In T. Mehner & K. Tockner (Eds.), *Encyclopedia of Inland Waters (Second Edition)* (pp. 155–162). Elsevier. https://doi.org/10.1016/B978-0-12-819166-8.00138-9
27. Igiehon, N. O. & Babalola, O. O. (2018). Rhizosphere Microbiome Modulators: Contributions of Nitrogen Fixing Bacteria towards Sustainable Agriculture. *International Journal of Environmental Research and Public Health*, *15* (4), Article 4. https://doi.org/10.3390/ijerph15040574
28. Islam, M. S., Kawasaki, H., Muramatsu, Y., Nakagawa, Y. & Seki, T. (2008). <I>*Bradyrhizobium iriomotense*</I> sp. Nov., Isolated from a Tumor-Like Root of the Legume <I>Entada koshunensis</I> from Iriomote Island in Japan. *Bioscience, Biotechnology, and Biochemistry*, *72*(6), 1416–1429. https://doi.org/10.1271/bbb.70739
29. Johnston, A. (2004). *Mechanisms and Regulation of Iron Uptake in the Rhizobia.* https://doi.org/10.1128/9781555816544.CH30
30. Josey, D. P., Beynon, J. L., Johnston, A. W. B. & Beringer, J. E. (1979). Strain Identification in *Rhizobium* Using Intrinsic Antibiotic Resistance. *Journal of Applied Bacteriology*, *46*(2), 343–350. https://doi.org/10.1111/j.1365-2672.1979.tb00830.x
31. Kathalia, M., Taryono, T. & Sayekti, R. S. (2020). The Response of Some Yardlong Bean (*Vigna unguiculata* subsp. *Sesquipedalis*) Accessions to Bradyrhizobium Inoculation. *Agrotechnology Innovation (Agrinova)*, *3*(1), Article 1. https://doi.org/10.22146/a.58347
32. Klepa, M. S., Helene, L. C. F., O´Hara, G. & Hungria, M. (2022). *Bradyrhizobium cenepequi* sp. Nov., *Bradyrhizobium semiaridum* sp. Nov., *Bradyrhizobium hereditatis* sp. Nov. And *Bradyrhizobium australafricanum* sp. Nov., symbionts of different leguminous plants of Western Australia and South Africa and definition of three novel symbiovars. *International Journal of Systematic and Evolutionary Microbiology*, *72*(7). https://doi.org/10.1099/ijsem.0.005446
33. Kumar, N., Lad, G., Giuntini, E., Kaye, M. E., Udomwong, P., Shamsani, N. J., Young, J. P. W. & Bailly, X. (2015). Bacterial genospecies that are not ecologically coherent: Population genomics of *Rhizobium leguminosarum*. *Open Biology*, *5*(1), 140133. https://doi.org/10.1098/rsob.140133
34. Leelahawonge, C., Nuntagij, A., Teaumroong, N., Boonkerd, N. & Pongsilp, N. (2010). Characterization of root-nodule bacteria isolated from the medicinal legume Indigofera tinctoria. *Annals of Microbiology*, *60*(1), Article 1. https://doi.org/10.1007/s13213-009-0005-8
35. Li, Y. H., Wang, R., Sui, X. H., Wang, E. T., Zhang, X. X., Tian, C. F., Chen, W. F. & Chen, W. X. (2019). *Bradyrhizobium nanningense* sp. Nov., *Bradyrhizobium guangzhouense* sp. Nov. And *Bradyrhizobium zhanjiangense* sp. Nov., isolated from effective nodules of peanut in Southeast China. *Systematic and Applied Microbiology*, *42*(5), 126002. https://doi.org/10.1016/j.syapm.2019.126002
36. Li, Y. H., Wang, R., Zhang, X. X., Young, J. Peter. W., Wang, E. T., Sui, X. H. & Chen, W. X. (2015). *Bradyrhizobium guangdongense* sp. Nov. And *Bradyrhizobium guangxiense* sp. Nov., isolated from effective nodules of peanut. *International Journal of Systematic and Evolutionary Microbiology*, *65*(Pt_12), 4655–4661. https://doi.org/10.1099/ijsem.0.000629
37. Liu, M., Adl, S., Cui, X., Tian, Y., Xu, X. & Kuzyakov, Y. (2020). In situ methods of plant-microbial interactions for nitrogen in rhizosphere. *Rhizosphere*, *13*, 100186. https://doi.org/10.1016/j.rhisph.2020.100186
38. Martins da Costa, E., Soares de Carvalho, T., Azarias Guimarães, A., Ribas Leão, A. C., Magalhães Cruz, L., de Baura, V. A., Lebbe, L., Willems, A. & de Souza Moreira, F. M. (2019). Classification of the inoculant strain of cowpea UFLA03-84 and of other strains from soils of the Amazon region as *Bradyrhizobium viridifuturi* (symbiovar tropici). *Brazilian Journal of Microbiology*, *50*(2), 335–345. https://doi.org/10.1007/s42770-019-00045-x
39. Miljakovic, D., Marinković, J., Ignjatov, M., Milosević, D., Nikolić, Z., Tintor, B. & Đukić, V. (2022). Competitiveness of *Bradyrhizobium japonicum* inoculation strain for soybean nodule occupancy. *Plant, Soil and Environment*, *68* (No. 1), 59–64. https://doi.org/10.17221/430/2021-PSE
40. Mohite, B. (2013). Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *Journal of Soil Science and Plant Nutrition*, *ahead*, 0–0. https://doi.org/10.4067/S0718-95162013005000051
41. Ngwenya, Z. D., Mohammed, M., Jaiswal, S. K. & Dakora, F. D. (2022). Phylogenetic relationships among *Bradyrhizobium* species nodulating groundnut (*Arachis hypogea* L.), jack bean (*Canavalia ensiformis* L.) and soybean (*Glycine max* Merr.) in Eswatini. *Scientific Reports*, *12*(1), 10629. https://doi.org/10.1038/s41598-022-14455-9
42. Noisangiam, R., Teamtisong, K., Tittabutr, P., Boonkerd,

N., Toshiki, U., Minamisawa, K. & Teaumroong, N. (2012). Genetic Diversity, Symbiotic Evolution, and Proposed Infection Process of *Bradyrhizobium* Strains Isolated from Root Nodules of *Aeschynomene americana* L. in Thailand. *Applied and Environmental Microbiology*, *78*(17), 6236–6250. https://doi.org/10.1128/AEM.00897-12

43. Nzoué, A., Miché, L., Klonowska, A., Laguerre, G., de Lajudie, P. & Moulin, L. (2009). Multilocus sequence analysis of bradyrhizobia isolated from *Aeschynomene* species in Senegal. *Systematic and Applied Microbiology*, *32* (6), 400–412. https://doi.org/10.1016/j.syapm.2009.06.002
44. Ojiewo, C. O., Janila, P., Bhatnagar-Mathur, P., Pandey, M. K., Desmae, H., Okori, P., Mwololo, J., Ajeigbe, H., Njuguna-Mungai, E., Muricho, G., Akpo, E., Gichohi-Wainaina, W. N., Variath, M. T., Radhakrishnan, T., Dobariya, K. L., Bera, S. K., Rathnakumar, A. L., Manivannan, N., Vasanthi, R. P., … Varshney, R. K. (2020). Advances in Crop Improvement and Delivery Research for Nutritional Quality and Health Benefits of Groundnut (*Arachis hypogaea* L.). *Frontiers in Plant Science*, *11*. https://www.frontiersin.org/articles/10.3389/fpls.2020.00029
45. Okubo, T., Fukushima, S., Itakura, M., Oshima, K., Longtonglang, A., Teaumroong, N., Mitsui, H., Hattori, M., Hattori, R., Hattori, T. & Minamisawa, K. (2013). Genome Analysis Suggests that the Soil Oligotrophic Bacterium Agromonas oligotrophica (*Bradyrhizobium oligotrophicum*) Is a Nitrogen-Fixing Symbiont of *Aeschynomene indica*. *Applied and Environmental Microbiology*, *79*(8), 2542–2551. https://doi.org/10.1128/AEM.00009-13
46. Payne, S. M. (1993). Iron acquisition in microbial pathogenesis. *Trends in Microbiology*, *1*(2), 66–69. https://doi.org/10.1016/0966-842X(93)90036-Q
47. Piromyou, P., Greetatorn, T., Teamtisong, K., Okubo, T., Shinoda, R., Nuntakij, A., Tittabutr, P., Boonkerd, N., Minamisawa, K. & Teaumroong, N. (2015). Preferential Association of Endophytic Bradyrhizobia with Different Rice Cultivars and Its Implications for Rice Endophyte Evolution. *Applied and Environmental Microbiology*, *81*(9), 3049–3061. https://doi.org/10.1128/AEM.04253-14
48. Piromyou, P., Greetatorn, T., Teamtisong, K., Tittabutr, P., Boonkerd, N. & Teaumroong, N. (2017). Potential of Rice Stubble as a Reservoir of *Bradyrhizobial* Inoculum in Rice-Legume Crop Rotation. *Applied and Environmental Microbiology*, *83*(22), e01488-17. https://doi.org/10.1128/AEM.01488-17
49. Piromyou, P., Songwattana, P., Boonchuen, P., Nguyen, H. P., Manassila, M., Tantanuch, W., Maikhunthod, B., Teamtisong, K., Tittabut, P., Boonkerd, N., Giraud, E. & Teaumroong, N. (2021). *The New Putative Type III Effector SkP48 in Bradyrhizobium sp. DOA9 is Involved in Legume Nodulation* [Preprint]. In Review. https://doi.org/10.21203/rs.3.rs-900464/v1
50. Piromyou, P., Songwattana, P., Greetatorn, T., Okubo, T., Kakizaki, K. C., Prakamhang, J., Tittabutr, P., Boonkerd, N., Teaumroong, N. & Minamisawa, K. (2015). The Type III Secretion System (T3SS) is a Determinant for Rice-Endophyte Colonization by Non-Photosynthetic *Bradyrhizobium*. *Microbes and Environments*, *advpub*, ME15080. https://doi.org/10.1264/jsme2.ME15080
51. Prell, J. & Poole, P. (2006). Metabolic changes of rhizobia in legume nodules. *Trends in Microbiology*, *14*(4), 161–168. https://doi.org/10.1016/j.tim.2006.02.005
52. Priyadarshini, P., Choudhury, S., Tilgam, J., Bharati, A. & Sreeshma, N. (2021). Nitrogen fixing cereal: A rising hero towards meeting food security. *Plant Physiology and Biochemistry*, *167*, 912–920. https://doi.org/10.1016/j.plaphy.2021.09.012
53. Puozaa, D. K., Jaiswal, S. K. & Dakora, F. D. (2017). African origin of *Bradyrhizobium* populations nodulating Bambara groundnut (*Vigna subterranea* L. Verdc) in Ghanaian and South African soils. *PLOS ONE*, *12*(9), e0184943. https://doi.org/10.1371/journal.pone.0184943
54. Rajendran, G., Sing, F., Desai, A. J. & Archana, G. (2008). Enhanced growth and nodulation of pigeon pea by co-inoculation of Bacillus strains with *Rhizobium* spp. *Bioresource Technology*, *99*(11), 4544–4550. https://doi.org/10.1016/j.biortech.2007.06.057
55. Rivas, R., Martens, M., de Lajudie, P. & Willems, A. (2009). Multilocus sequence analysis of the genus *Bradyrhizobium*. *Systematic and Applied Microbiology*, *32*(2), 101–110. https://doi.org/10.1016/j.syapm.2008.12.005
56. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning. A laboratory Manual*. Cold Spring Harbor Laboratory Press. https://is.muni.cz/publication/372112/en/Molecular-Cloning-A-laboratory-Manual/Sambrook-Fritsch-Maniatis
57. Shiriki, D., Igyor, M. A. & Gernah, D. I. (2015). Nutritional evaluation of complementary food formulations from maize, soybean and peanut fortified with Moringa oleifera leaf powder. *Food and Nutrition Sciences*, *6*(05), 494.
58. Silva, F. V., De Meyer, S. E., Simões-Araújo, J. L., Barbé, T. da C., Xavier, G. R., O'Hara, G., Ardley, J. K., Rumjanek, N. G., Willems, A. & Zilli, J. E. (2014). *Bradyrhizobium manausense* sp. Nov., isolated from effective nodules of *Vigna unguiculata* grown in Brazilian Amazonian rainforest soils. *International Journal of Systematic and Evolutionary Microbiology*, *64*(Pt_7), 2358–2363. https://doi.org/10.1099/ijs.0.061259-0
59. Somasegaran, P. & Hoben, H. J. (2012). *Handbook for Rhizobia: Methods in Legume-Rhizobium Technology*. Springer Science & Business Media.
60. Songwattana, P., Chaintreuil, C., Wongdee, J., Teulet, A., Mbaye, M., Piromyou, P., Gully, D., Fardoux, J., Zoumman, A. M. A., Camuel, A., Tittabutr, P., Teaumroong, N. & Giraud, E. (2021). Identification of type III effectors modulating the symbiotic properties of *Bradyrhizobium vignae* strain ORS3257 with various *Vigna* species. *Scientific Reports*, *11*(1), Article 1. https://doi.org/10.1038/s41598-021-84205-w
61. Songwattana, P., Noisangiam, R., Teamtisong, K., Prakamhang, J., Teulet, A., Tittabutr, P., Piromyou, P., Boonkerd, N., Giraud, E. & Teaumroong, N. (2017). Type 3 Secretion System (T3SS) of *Bradyrhizobium* sp. DOA9 and Its Roles in Legume Symbiosis and Rice Endophytic Association. *Frontiers in Microbiology*, *8*. https://www.frontiersin.org/articles/10.3389/fmicb.2017.01810
62. Stacey, G., Burris, R. H. & Evans, H. J. (1992). *Biological Nitrogen Fixation*. Springer Science & Business Media.
63. Sturz, A. V., Christie, B. R., Matheson, B. G. & Nowak, J. (1997). Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. *Biology and Fertility of Soils*. https://scholar.google.com/scholar_lookup?

title=Biodiversity+of+endophytic+bacteria+which+colonize+red+clover+nodules%2C+roots%2C+stems+and+foliage+and+their+influence+on+host+growth&author=Sturz%2C+A.V.&publication_year=1997

64. Traxler, M. F., Seyedsayamdost, M. R., Clardy, J. & Kolter, R. (2012). Interspecies modulation of bacterial development through iron competition and siderophore piracy. *Molecular Microbiology*, *86*(3), 628–644. https://doi.org/10.1111/mmi.12008
65. Van Rossum, D., Schuurmans, F. P., Gillis, M., Muyotcha, A., Van Verseveld, H. W., Stouthamer, A. H. & Boogerd, F. C. (1995). Genetic and phenetic analyses of *Bradyrhizobium* strains nodulating peanut (*Arachis hypogaea* L.) roots. *Applied and Environmental Microbiology*, *61*(4), 1599–1609. https://doi.org/10.1128/aem.61.4.1599-1609.1995
66. Versalovic, J., Schneider, M., Bruijn, F. J. D. & Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in Molecular and Cellular Biology*, *5*(1), 25–40.
67. White, J., Prell, J., James, E. K. & Poole, P. (2007). Nutrient Sharing between Symbionts. *Plant Physiology*, *144*(2), 604–614. https://doi.org/10.1104/pp.107.097741
68. Wójcik, M., Kalita, M. & Małek, W. (2019). Numerical analysis of phenotypic properties, genomic fingerprinting, and multilocus sequence analysis of *Bradyrhizobium* strains isolated from root nodules of *Lembotropis nigricans* of the tribe Genisteae. *Annals of Microbiology*, *69*(11), 1123–1134. https://doi.org/10.1007/s13213-019-01491-6
69. Yates, R. J., Howieson, J. G., Reeve, W. G. & O'Hara, G. W. (2011). A re-appraisal of the biology and terminology describing rhizobial strain success in nodule occupancy of legumes in agriculture. *Plant and Soil*, *348*(1), 255. https://doi.org/10.1007/s11104-011-0971-z
70. You, Z., Marutani, M. & Borthakur, D. (2002). Diversity among *Bradyrhizobium* isolates nodulating yardlong bean and sunnhemp in Guam. *Journal of Applied Microbiology*, *93*(4), 577–584. https://doi.org/10.1046/j.1365-2672.2002.01733.x
71. Young, C. C. & Chao, C. C. (1989). Intrinsic antibiotic resistance and competition in fast- and slow-growing soybean rhizobia on a hybrid of Asian and US cultivars. *Biology and Fertility of Soils*, *8*(1), 66–70. https://doi.org/10.1007/BF00260518
72. Yu, X., Shoaib, M., Cheng, X., Cui, Y., Hussain, S., Yan, J., Zhou, J., Chen, Q., Gu, Y., Zou, L., Zhang, X., Hao, S., Zhao, K., Ma, M., Xiang, Q., Li, S. & Zou, T. (2022). Role of rhizobia in promoting non-enzymatic antioxidants to mitigate nitrogen-deficiency and nickel stresses in *Pongamia pinnata*. *Ecotoxicology and Environmental Safety*, *241*, 113789. https://doi.org/10.1016/j.ecoenv.2022.113789
73. Yuhashi, K.-I., Ichikawa, N., Ezura, H., Akao, S., Minakawa, Y., Nukui, N., Yasuta, T. & Minamisawa, K. (2000). Rhizobitoxine Production by *Bradyrhizobium elkanii* Enhances Nodulation and Competitiveness on *Macroptilium atropurpureum*. *Applied and Environmental Microbiology*, *66*(6), 2658–2663. https://doi.org/10.1128/AEM.66.6.2658-2663.2000