SELECTION OF BRADYRHIZOBIA FOR PEANUT PRODUCTION IN THE LAO PEOPLE'S DEMOCRATIC REPUBLIC

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การคัดเลือกแบรดดี้ไรโซเบียมเพื่อการผลิตถั่วลิสงในสาธารณรัฐประชาธิปไตยประชาชนลาว

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ต่านี่ พิมพ์พงศ์ : การคัดเลือกแบรดดี้ไรโซเบียมเพื่อการผลิตถั่วลิสงในสาธารณรัฐประชาธิปไตยประชาชนลาว (SELECTION OF BRADYRHIZOBIA FOR PEANUT PRODUCTION IN THE LAO PEOPLE'S DEMOCRATIC REPUBLIC) อาจารย์ที่ปรึกษา : ศาสตราจารย์ ดร. หนึ่ง เตียอำรุง, 94 หน้า

คำสำคัญ: แบรดดี้ไรโซเบียม/ถั่วลิสง (*Arachis hypogea* L.)/การตรึงไนโตรเจน

แบรดดี้ไรโซเบียม *Bradyrhizobium* จัดเป็น Alphaproteobacteria ที่อยู่ในวงศ์ Bradyrhizobiaceae มีความสามารถในการตรึงในโตรเจนจากอากาศ ให้อยู่ในรูปของแอมโมเนียที่พืชในตระกูลถั่วสามารถนำไปใช้ประโยชน์ได้ อย่างไรก็ตามปฏิสัมพันธ์ระหว่างพืชตระกูลถั่วและแบรดดี้ไรโซเบียมนั้นถูกจำกัดด้วยความจำเพาะเจาะจงของชนิดและสายพันธุ์ถั่ว ดังนั้น การคัดเลือกสายพันธุ์ของแบรดดี้ไรโซเบียมเพื่อนำไปใช้ เป็นปุ๋ยชีวภาพสำหรับพืชตะกูลถั่วจึงเป็นสิ่งที่จำเป็น ถั่วลิสง (*Arachis hypogea* L.) เป็นพืชตระกูลถั่วที่ได้รับความนิยมและมีการปลูกมากที่สุดในประเทศ สปป. ลาว อย่างไรก็ตามการประยุกต์ใช้ปุ๋ยชีวภาพนี้ยังไม่ได้รับความสนใจมากนัก ดังนั้น วัตถุประสงค์ของการศึกษานี้คือ เพื่อคัดเลือกสายพันธุ์ของแบรดดี้ไรโซเบียมที่เหมาะสม มีประสิทธิภาพสูงในการเพิ่มความสามารถในการผลิตถั่วลิสงสำหรับพื้นที่ในประเทศ สปป. ลาว โดยคัดแยกเชื้อแบรดดี้ไรโซเบียม 14 สายพันธุ์ PMVTL-01, PMVTL-02, SMVTL-01, SMVTL-02, SPXBL-01, SPXBL-02, SPXBL-03, SPXBL-04, SPXBL-05, SPXBL-06, SPXBL-07, BLXBL-01, BLXBL-02 และ BLXBL-03 จากปมรากถั่วลิสง ที่ได้จากตัวอย่างดินจากพื้นที่ต่างๆ ของประเทศ สปป. ลาว สายพันธุ์ PMVTL-01, SMVTL-02 และ BLXBL-03 เป็นเชื้อ 3 ลำดับแรกที่มีประสิทธิภาพสูงที่สุดในการเพิ่มความสามารถในการเจริญเติบโตของถั่วลิสง โดยทั้ง 3 สายพันธุ์นี้ได้ถูกจำแนกเพิ่มเติมได้แก่ การวิเคราะห์ลักษณะทางกายภาพ ชีวเคมี ความสามารถต้านทานสารปฏิชีวนะ และการตรวจวิเคราะห์ลำดับนิวคลีโอไทด์โดยเทคนิค multilocus DNA sequencing ของยีน 16S rRNA และ 4 ยีนพื้นฐานได้แก่ *recA*, *atpD*, *glnII* และ *rpoB* พบว่าแบคทีเรียสายพันธุ์เหล่านี้เป็นแบคทีเรียในสกุล แบรดดี้ไรโซเบียม ซึ่งสายพันธุ์ PMVTL-01 และ SMVTL-02 มีความใกล้เคียงกับแบรดดี้ไรโซเบียมในกลุ่มของ *Bradyrhizobium* genosp. SA-3 Rp7b และ *B. zhanjiangense* ตามลำดับ ในขณะที่สายพันธุ์ BLXBL-03 ใกล้เคียงกับ *Bradyrhizobium* sp. CCBAU51745 และ *B. manausense* BR3351 ความสามารถในการแข่งขันเข้าสร้างปมของสายพันธุ์เหล่านี้ทำการทดสอบโดยเปรียบเทียบกับแบรดดี้ไรโซเบียมสายพันธุ์ SUTN9-2::GFP ผลการทดลองพบว่าสายพันธุ์ SMVTL-02 เท่านั้นที่มีความสามารถในการเข้าสร้างปมสูงกว่าแบรดดี้ไรโซเบียมสายพันธุ์ SUTN9-2::GFP ที่อัตราส่วนของสายพันธุ์ SUTN9-2::GFP ต่อ แบรดดี้ไรโซเบียมสายพันธุ์ SMVTL-02 ในสัดส่วน 1:9 พบว่า การเข้าสร้างปม ของสายพันธุ์ SUTN9-2::GFP อยู่ที่ (27.93% SUTN9-2::GFP และ 72.07% SMVTL-02)

ซึ่งในสัดส่วน 3:7 การเข้าสร้างปมอยู่ที่ (28.84% SUTN9-2::GFP และ 71.16% SMVTL-02) ในสัดส่วน 5:5 การเข้าสร้างปมอยู่ที่ (38.73% SUTN9-2::GFP และ 61.27% SMVTL-02) ในสัดส่วน 7:3 การเข้าสร้างปมอยู่ที่ (36.82% SUTN9-2::GFP และ 63.18% SMVTL-02) และในสัดส่วน 9:1 การเข้าสร้างปมอยู่ที่ (64.24% SUTN9-2::GFP และ 35.76% SMVTL-02) จากนั้นได้ทดสอบความสามารถในการเข้าสร้างปมของแบรดดี้ไรโซเบียมสายพันธุ์ SMVTL-02 ในระดับกระถาง ด้วยตัวอย่างดินจาก สปป. ลาว โดยใช้การติดตามโดย แบรดดี้ไรโซเบียมสายพันธุ์ SMVTL-02 ที่มียีนในการติดตาม *DsRed* ผลการทดลองแสดงให้เห็นว่าสายพันธุ์ที่มีการแสดงออกของยีนที่มีการติดตามด้วยยีน *DsRed* มีความสามารถในการเข้าสร้างปมมากกว่าสายพันธุ์ตามธรรมชาติในดิน โดยสายพันธุ์ SMVTL-02::DsRed มีความสามรถในการเข้าสร้างปม 81.98% ขณะที่สายพันธุ์ประจำถิ่น อยู่ที่ 18.02% นอกจากนี้ผลการทดลองยังแสดงให้เห็นว่า Acetylene Reduction Assay (ARA) จำนวนปม น้ำหนักแห้งปม และน้ำหนักแห้งทั้งหมดของถั่วลิสง ที่ทำการทดลองปลูกในระดับกระถางร่วมกับการใช้ปุ๋ยชีวภาพ สายพันธุ์ SMVTL-02::DsRed มีศักยภาพในการส่งเสริมการเจริญเติบโตของถั่วลิสงสูงกว่าการไม่ใช้ปุ๋ยชีวภาพ ดังนั้นแบรดดี้ไรโซเบียมสายพันธุ์ SMVTL-02 จะถูกนำไปทดสอบในระดับแปลงต่อไป เพื่อใช้เป็นหัวเชื้อปุ๋ยชีวภาพในการผลิตถั่วลิสงใน สปป. ลาว

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2565

ลายมือชื่อนักศึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____
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TARNEE PHIMPHONG : SELECTION OF BRADYRHIZOBIA FOR PEANUT PRODUCTION IN THE LAO PEOPLE'S DEMOCRATIC REPUBLIC. THESIS ADVISOR : PROF. NEUNG TEAUMROONG, Ph.D., 94 PP.

Keyword: *Bradyrhizobium* sp./*Arachis hypogea* L./Nitrogen fixation

Bradyrhizobium sp. is Alphaproteobacteria bacteria (Family: Bradyrhizobiaceae) that can promote leguminous growth by fix N_2 and convert to bioavailable ammonium. However, the interaction between leguminous plant and *Bradyrhizobium* is limited and known as host specificity. Therefore, selection of an appropriate bradyrhizobial strain for use as biofertilizer inoculum for legume is necessary. Peanut (*Arachis hypogea* L.) is the most popular of legume production in Lao People's Democratic Republic (PDR). However, applications of biofertilizer have not been promoted. Therefore, the objective in this research was to obtain the appropriate bradyrhizobial strain which provides high efficiency in peanut production in the Lao PDR. The 14 strain bradyrhizobial isolates PMVTL-01, PMVTL-02, SMVTL-01, SMVTL-02, SPXBL-01, SPXBL-02, SPXBL-03, SPXBL-04, SPXBL-05, SPXBL-06, SPXBL-07, BLXBL-01, BLXBL-02 and BLXBL-03 were isolated from root nodules of *A. hypogea* L. trapped with Lao PDR soil samples. The isolates PMVTL-01, SMVTL-02 and BLXBL-03 were the top three showing high efficiency for peanut growth promotion. The top three isolates were characterized along with phenotypic, biochemical, physiological characteristics, antibiotic resistance profiles and multilocus sequence analysis of the 16S rRNA gene and four housekeeping genes (*recA*, *atpD*, *glnII*, and *rpoB*). The isolates were assigned to the genus *Bradyrhizobium* strains PMVTL-01 and SMVTL-02 were closely related with *Bradyrhizobium* genosp. SA-3 Rp7b and *B. zhanjiangense*, respectively, whilst strain BLXBL-03 was closely related with *Bradyrhizobium* sp. CCBAU51745 and *B. manausense* BR3351. The competitiveness of these strains with *Bradyrhizobium* sp. strain SUTN9-2::GFP was analyzed, the results indicated that only *Bradyrhizobium* sp. strain SMVTL-02 performed a number of occupied nodules higher than SUTN9-2::GFP, at 1:9 (27.93% SUTN9-2::GFP and 72.07% SMVTL-02), 3:7 (28.84% SUTN9-2::GFP and 71.16% SMVTL-02), 5:5 (38.73% SUTN9-2::GFP and 61.27% SMVTL-02), 7:3 (36.82% SUTN9-2::GFP and 63.18% SMVTL-02) and 9:1 (64.24% SUTN9-2::GFP

and 35.76% SMVTL-02). In addition, the competitiveness of selected strain *Bradyrhizobium* sp. strain SMVTL-02 in soil samples from Lao PDR in pot level was employed by tagging the SMVTL-02 with *DsRed* gene. The results demonstrated the DsRed-expressing tagged strain was higher in nodules occupancy than indigenous strains (81.98% SMVTL-02::DsRed and 18.02% indigenous). Moreover, the results of Acetylene Reduction Assay (ARA), nodules number, nodules dry weight and total plant dry weight from the pot experiment which was inoculated with SMVTL-02::DsRed strain indicated higher potential to promote peanut growth than non-inoculation. Thus, *Bradyrhizobium* sp. strain SMVTL-02 will be tested in the field prior to produce as biofertilizer inoculum for peanut production in Lao PDR.

School of Biotechnology
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Student's Signature _____
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Tarnee Phimphong

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

PDR	=	Lao People's Democratic Republic
PAFO	=	Provincial Agriculture and Forestry Office
SPXBL	=	Sanphon village, Xayaboury province, Lao PDR
BLXBL	=	Buamlao village, Xayaboury province, Lao PDR
PMVTL	=	Parkxarb mai village, Vientiane capital, Lao PDR
SMVTL	=	Somsamai village, Vientiane capital, Lao PDR
USA	=	United States of America
K	=	kilo (000)
$	=	BUSD
LAK	=	Lao PDR kip
ARA	=	acetylene Reduction Assay
ADP	=	adenosine 5′- diphosphate
C	=	carbon
O_2	=	oxygen
N_2	=	nitrogen grass
Pi	=	phosphate (inorganic)
DNA	=	deoxyribonucleic acid
RNA	=	ribonucleic acid
IAA	=	indole acetic acid
PCR	=	Polymerase Chain Reaction
LB	=	Luria-Bertani medium
YM	=	yeast extract-mannitol medium
HM	=	HEPES-MES medium
˚C	=	Degree Celsius
pH	=	potential of hydrogen ion
α	=	Alpha
β	=	Beta
%	=	Percent

LIST OF ABBREVIATIONS (Continued)

L	=	liter
g	=	gram
mg	=	milligram
min	=	minute
ml	=	milliliter
mM	=	millimole
M	=	molar
µM	=	micromolar
µm	=	micrometer
µmol	=	micromole
µg	=	microgram
µl	=	microliter
kg	=	kilogram
cm	=	centimeters
Ha	=	hectare
h	=	hour
ha	=	hectare
SD	=	standard Deviation
OD	=	optical density
rpm	=	revolution per minute
W/V	=	%weight per volume
V/V	=	volume per volume
GFP	=	green fluorescent protein
DsRed	=	red fluorescent protein

CHAPTER I
INTRODUCTION

1.1 Significance of study

Nitrogen (N) is the primary limiting nutrient in most terrestrial ecosystems, it is a nutrient supplied to most plants and a determinant of plant growth (Hodge et al., 2000). In addition, nitrogen is a component of proteins, enzymes, chlorophyll, and plant growth regulators, nutrient deficiencies resulted in decreased growth, yellowing of leaves, and reduced branching and small trifoliate leaves in legumes (D. W. Wolfe et al., 1988; Martins et al., 1997; Ougham et al., 2005). *Bradyrhizobium* is gram-negative bacteria, capable of symbiosis with leguminous plants (Kuykendall, 2015). *Bradyrhizobium* induces the development of root nodules in leguminous plants and converts atmospheric di-nitrogen to ammonia using the nitrogen-fixing enzyme called nitrogenase (de Bruijn, 2015). 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 (Wilkinson et al., 1996). 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). The 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 who are 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 bio-fertilizer (Phommalath, 2018). 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. Therefore, an appropriate bradyrhizobial strain for peanut growth promotion in the Lao PDR is required. The isolation was conducted using soil samples from the Lao PDR with the peanut trapping technique. The strain characterizations were employed using a polyphasic approach. The evaluation of strain competitiveness was compared among Thai bradyrhizobia commercial strains and indigenous strains in the Lao PDR soil sample.

1.2 Research objective

1.2.1 Main objective

To select the most effective bradyrhizobial strain which gives high efficiency for peanuts growth-promotion in the Lao People's Democratic Republic.

1.2.2 Specific objectives

1) To isolate bradyrhizobial strains from Lao PDR soil.

2) To select the higher efficiency strains or equivalent when compared with commercial strains TAL173 and SUTN9-2.

3) To identify and characterize of selected strains.

4) To investigate the strain competitiveness with commercial and indigenous strains.

CHAPTER II

LITERATURE REVIEW

2.1 Legume production in Laos PDR

Legumes are one of the main cash crops after rice and maize for the Lao PDR economy due to high local and external demand (Table 2.1). In data, main legume production such as peanut cover area 18,887 ha with product of 49,105 tons, soybean cover area 4,260 ha with product of 7,960 tons and dry bean cover area 2,520 ha with product of 4,475 tons (FAOSTAT, 2019). Therefore, such crops are more important, the species grown for food and fresh consumption include *Glycine max* L. Merr., *Vigna radiata* L. Wilezek, *V. unguiculata* spp. *sesquipedalis*, *Cajanus cajan*, *Phaseolus vulgaris* L., *Arachis hypogaea* L. From previous reports, farmers in Northern Lao PDR supplementing pigs with forage legumes *Stylosanthes guianensis* CIAT 184 (Stylo 184) (Kongpanh et al., 2010; Peters et al., 2008; Stur & Kopinski, 2010). The legume seeds can be found all over Laos, from north to south, some of them are "local varieties" or others have been suggested because of a lack of information regarding classification. The peanut production systems and sowing techniques used by farmers differ in the North and the South. Peanuts, mung beans and soybeans can be found along the riverbanks or islands which are typically planted with cereal crops, also in the highland farmers grow these crops, but on a small scale. Large-area production of legumes and seeds occurs after the main crop. The sowing period is between the middle and the end of the rainy season, and the harvest is at the beginning of the dry season. Legumes are also grown along the banks in the dry season (Kongpanh et al., 2010). Moreover, the legumes were used as crop location or crop mixed systems in Lao PDR (Lienhard et al., 2020; LMAF, 2014). Legume production in Laos PDR was 31,108 tons in 2019 and is expected to change by an average of 6.44%, the country has approximately 18,907 hectares under bean cultivation (Selina Wamucii, 2019).

In Lao PDR, the main of Legume production as for export. In Xayaboury province, agricultural products are becoming more and more important. In addition,

rice is produced in every village and exported in large quantities to other countries, Thailand, and China. Thailand also has an increasing demand for maize, peanuts, soybeans, and sesame. According to statistics provided by PAFO in 2004, more than 14,000 tons of maize, with an export value of USD 1.0 million, 3,200 tons of peanut (USD 780,000), 1,040 tons of Job's tears (USD 156,000) and 720 tons of sesame (USD 430,000) where produced in the five northern districts (Helberg et al., 2005). In Salavanh province, the total production area of legume (peanut) is 799.6 hectares, yield 939.48 tons. The volume of legume (peanut) export was increasing in every year since 2008 to 2013, from LAK 32,886.8 million to LAK 88,171.9 million in (Table 2.2) (LMAF, 2014). Export volume of Lao legume, in 2019 Lao PDR exported 3,724 tons of legume. For the year 2019 alone, demand for Lao legume increased by 215.059% compared to 2018 during 2017. In 2019, legume exports grew by 132.17 percent, with exporters' net 5.61 USD m for 2019 (Selina Wamucii, 2019). In 2020, Laos exported $386k of dried legumes, making it the 111th largest exporter of dried legumes in the world, that same year dried legumes are Laos' 177th largest export. The main destinations for Lao dried legumes exports are Thailand ($378k), Chinese Taipei ($7.98k) and Turkey ($851). The fastest growing Lao dry legume export markets between 2019 and 2020 are Taipei ($7.07k) and Turkey ($851). On the other hand, in 2020, Laos imported $2.03M of dried legumes and became the 132nd largest importer of dried legumes in the world, that same year dried legumes are the 337th largest import of Laos. Laos imports most of the dried legumes from Thailand ($714k), Brazil (658k), USA (407k), Vietnam (246k) and France ($724). The fastest growing import markets in dry legumes for Laos between 2019 and 2020 are Brazil ($658k), USA ($$302k) and Vietnam ($56.6k) (OEC, 2020).

The information of biofertilizer in Lao PDR, in seven biofertilizer factories were in established in 1990, this led to an increase in biofertilizer production levels to approximately 2,000 tons by 2004 and the products being promoted as a tool "Chemical-Free agriculture". Nevertheless, these products did not prove to have a significant impact, and interest in technology has decreased. From then, a bit of information about the prospects and developments of biofertilizers in Lao PDR because of many people (farmer) confuse between biofertilizer and organic fertilizer (compost) and most people cannot produce biofertilizer, only research institute can

do. But there is a new local company named Gaia Vita in 2020, working with the group France's Biopost-Cofuna has begun selling a new biofertilizer made from local organic matter this is compost if biofertilizer must identify micro used. Interestingly, the demand increases of biofertilizers in the coming years in Lao PDR (Atieno et al.,2020; Roder et al., 2005) However, the legume production in Lao PDR, the smallholder farmers grow crops without inoculation and the lowest of use fertilizer. Because chemical fertilizers are expensive and not immediately available resulting in very low productivity. The main limitations in bio-fertilizer production are the lack of qualified personnel and production capacity. The farmers and distributors are often less enthusiastic or aware of the importance of this technology. As a result, the supply and use of biofertilizers are low (Atieno et al., 2020; Win et al., 2002; Rao et al., 2011).

Table 2.1 Lao National Agricultural Production data (2007) (Kongpanh et al., 2010b).

Crop	Area (ha)	Yield (T/ha)	Production (T)
Rice	604,147	3.63	2,193,400
Upland Rice	105,696	1.77	187,450
Irrigated	71,400	4.61	329,200
Maize	154,255	4.48	690,795
Peanut	15,965	1.96	35,070
Soybean	8,040	1.30	10,455
Mungbean	2,450	1.00	2,470
Vegetable & Bean	84,335	-	734,385

Table 2.2 Production and trade of peanut in Salavanh (LMAF, 2014).

Year	Production area (Ha)	Export (Ton)	Million (LAK)
2008	6,746.0	5,059.5	32,886.8
2009	6,790.0	6,111.0	41,554.8
2010	6,790.0	7,638.8	53,471.3
2011	7,125.0	8,015.6	57,712.5
2012	8,122.0	10,355.6	75,595.5
2013	8,250.0	11,756.3	88,171.9

2.2 Importance of nitrogen

Although nitrogen is one of the most abundant elements in the world, However, it is an important limiting element for most plant growth as it is indispensable (Graham & Vance, 2000; Smil, 1999; Socolow, 1999). Nitrogen as nutrient that essential to plant function, the ammonium and nitrates absorbed by plants are converted to the amino N component of the amino acid, which is a key building block of plant proteins, nucleic acids, coenzymes, various secondary metabolites and chlorophyll (Foyer, 2018). Plants obtain nitrogen from two main sources: (1) from commercial fertilizers, manure, or mineralization of organic matter; and (2) from the atmosphere through symbiotic N_2 fixation, the N_2 is strong chemical stability so which makes it inaccessible to most organisms including plants, nevertheless only some prokaryotic microorganisms are able to fix nitrogen that is destroy triples to covalent bonds between nitrogen atoms and produce ammonium (Shridhar, 2012; Vance, 2001).

2.3 Peanut or groundnut

Peanuts (*Arachis hypogea* L.) commonly known as peanut or groundnut belong to the family Leguminosae, the subfamily Papilionoideae, and the dalbergioid clade. The genus *Arachis* species were divided into two subspecies and six species (Doyle & Luckow, 2003; Fabra et al., 2010; Lavin et al., 2001). Peanut is one of the most legumes globally widespread (Rami et al., 2014). Peanut is important food crops grown to produce oil, protein and has a high energy value, the production of peanuts worldwide is mainly devoted to oils and food products, from 1996 to 2000, 49% of peanuts were used to produce oil, and 41% were components of food products (Akram et al., 2018; Rami et al., 2014). Peanut by-products contain other functional compounds such as fat 40–50%, protein 20–50%, carbohydrates 10–20%, fiber, polyphenols, antioxidants, vitamins, niacin, folacin, calcium, phosphorus, magnesium, iron, zinc, riboflavin, potassium, thiamine, etc which can be added as functional ingredients in many processed foods. Thus, peanut and peanut oil protein food is an important part of the world trade in oilseeds and products. The fifth most important oilseed in the world due to production volume ranks fifth

after soybean oil, sunflower oil, colza oil, and palm oil (Arya et al., 2016; Fabra et al., 2010). The largest producers in the world are China, India, Nigeria, the United States, and Myanmar, respectively. China can produce up to 37% peanuts, Africa 25%, India 21%, America 8%, and Oceania 6%. India is the main export, which accounts for 37% of global exports, Argentina 13%, the United States 10%, China 8%, and Malawi for 5%. Also, the major of importers are Netherlands 17%, Indonesia 10%, Mexico 7%, Germany 6%, and Russia 5% of the world imports respectively (FAOSTAT, 2013). This plant can grow intercropping, sorghum, maize and other grains due to its N_2 fixation ability, drought tolerance and shade tolerance (Y. F. Zhang et al., 2008).

Traditionally, the peanut plants have been reported to be effective nodulated with slow-growing rhizobia belonging to the genus *Bradyrhizobium*, morphological and molecular analysis of peanut symbionts from different regions demonstrates a high level of heterogeneity and diversity. The study on the rhizobia diversity of peanut rhizobia in China soils made it possible to group bradyrhizobia not just according there to geographical but also to their cultivar origin (X. Zhang et al., 1999). Based on the analysis of the 16S and 16-23S ITS RFLP and 16S rDNA sequences, Chinese isolated strains were phylogenetically related to *B. liaoningense*, *B. japonicum* and *B. yuanmingense* (J. K. Yang & Zhou, 2008). Rhizobia inoculation improved the nitrogen fixation of peanuts. However, the effectiveness of symbiosis was influenced by competition between rhizobia and native rhizobia. Therefore, the selection of species with the ability to modulate nitrogen fixation and win the competition with traditional breeds is very important (Tauriani et al., 2002).

2.4 *Rhizobium* and *Bradyrhizobium*

Rhizobia are the generic name for Alphaproteobacteria and Betaproteobacteria Gram-negative groups. Some groups can form nodules on the roots or in some cases on the host stem and fix nitrogen in legume host symbiosis (Garrity et al., 2005; J. I. Sprent, 2008). The rhizobium genus is the first group of these bacteria to be described and that why this name is often used for nitrogen-fixing bacteria in legumes. Currently, the most known rhizobia belong to alphaproteobacterial in the family *Rhizobiaceae* (*Rhizobium*, *Ensifer* (*syn. Sinorhizobium*)), *Phyllobacteriaceae*

(*Mesorhizobium*, *Aminobacter*, *Phyllobacterium*), *Allorhizobium*, *Brucellaceae* (*Ochrobactrum*), *Pararhizobium*, *Neorhizobium*, *Shinella*, *Methylobacteriaceae* (*Methylobacterium*, *Microvirga*), *Hyphomicrobiaceae* (*Devosia*), *Xanthobacteraceae* (*Azorhizobium*) and *Bradyrhizobiaceae* (*Bradyrhizobium*) but some species belong to the betaproteobacteria in the family *Burkholderiaceae* (*Paraburkholderia*, *Cupriavidus*, *Trinickia*) (Andrews & Andrews, 2017; de Lajudie et al., 2019; Estrada-de los Santos et al., 2018; Mousavi, 2016).

Bradyrhizobium is gram-negative bacilli (rod-shaped) soil bacteria that can infect and form nodules on root plants. Also, they have symbiotic relationships with the leguminous plant, which cannot live without these bacteria's essential nitrogen-fixing processes. *Bradyrhizobium* is generally slow growing when compared with another rhizobium, nitrogen fixing bacteria that forms a symbiotic relationship with legumes plants, can found in roots or rhizosphere, where they cause the formation of nodules (Mpepereki et al., 1996; Somasegaran & Hoben, 2012). There are many species of *Bradyrhizobium* (Family: Bradyrhizobiaceae) *B. betae*, *B. canariense*, *B. elkanii*, *Bradyrhizobium genosp*, *B. japonicum*, *B. liaonigense*, *B. lupini*, *B. yuanmingense*, *B. manausense*, *B. guangdongense* and *B. guangxiense* (Li et al., 2015; Marcondes De Souza et al., 2013). In addition, there are many plant hosts groups of legumes could be nodulated by Bradyrhizobium sp. such as *Cajanus cajan*, *Arachis hypogaea*, *Acacia mearnsii*, *A. mangium*, *A. auriculiformis*, *Phaseolus lunatus*, *Psophocarpus tetragonoloba, Macroptilium atropurpureum*, *C. tetragonolobus*, *Vigna* spp., *Desmodium* spp., *Stylosanthes* spp. and *Lablab purpureus* (Somasegaran & Hoben, 2012).

2.5 Interaction and Nitrogen fixation

Many species of the rhizobia are diazotrophs, they fixed atmospheric nitrogen and are symbiotic with plant roots exceptionally in legume plants. The symbiotic nitrogen-fixing interaction between rhizobia and legumes is a symbiotic relationship in which both plants and bacteria benefit. Biological nitrogen fixation, this reaction is appeared by the activity of the nitrogenase enzyme, which converts atmospheric nitrogen into ammonia and can use as a nitrogen source for plants (Deacon, 1997). Rhizobia are hosted and supplied carbon sourced by legumes and on the other

hand legumes obtain ammonia from rhizobia (Clúa et al., 2018; Lindström & Mousavi, 2020), the model of interaction between rhizobia and legumes is displayed in Fig.2.1, 2.2 and 2.4.(a).

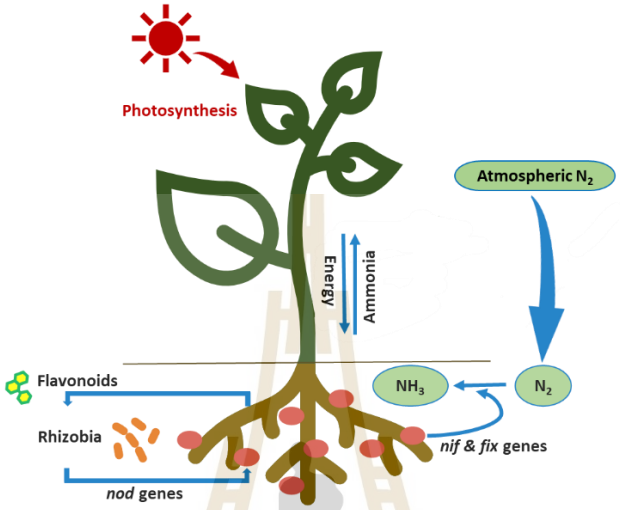

Figure 2.1 Summary model of interactions between rhizobia and legumes. Signal exchange in interactions starts from flavonoids from legume roots induce protein transcription factor Nodulation D (NodD) in rhizobium for compatible rhizobia (Lindström & Mousavi, 2020).

Nodulation of legumes by rhizobia is a complex and interesting developmental phenomenon, this requires a series of biochemical interactions between the bacteria and the host (Brewin, 1991; Fisher & Long, 1992; Long, 1989; Long & Staskawicz, 1993; Rolfe & Gresshoff, 1988; VERMA & S, 1983). In the process of this relationship, the roots plant sent the signal to bacteria and alters the growth of epidermal hairs on the root surface to curl them. Then bacteria induce cell divisions in the normal cells of the inner cortex of the plant root, which leads to the formation of nodular tissue. Bacteria trapped in the roots of the curled hair cause the strands to become infected, which is a bulb that comes from plants this penetrates the outer vegetative cells while the bacteria proliferate within them. As the nodule develops, the infection thread expands and penetrates each target cell (Hirsch et al., 1982). Bacteria there are surrounded by a plant-derived membrane and then released into

the cytoplasm of these cells, when released bacteria differentiate into a morphologically altered form known as bacteroid and begin to synthesize nitrogenase and other proteins required for nitrogen fixation. Plant cells also differentiate and express many proteins called nodulins, such as leghemoglobin, biological interactions result in a reduction of atmospheric dinitrogen to ammonia by bacteroid which the host plant is then utilized (González & Marketon, 2003). In this interaction, the bacteria are internalized to endosymbionts within the cells of the embryonic root organ, the nodule. The formation of a functional nitrogen fixation nodule depends on two separate processes. But there is a highly coordinated infection by rhizobia and organogenesis, which occur in the epidermal and cortical layers (Clúa et al., 2018). The reaction was of biological nitrogen fixation (BNF) showed below:

$$N_2 + 8H^+ + 8e^- + 16ATP \xrightarrow{\text{nitrogenase}} 2NH_3 + H_2 + 16ADP + 16Pi$$

nitrogen (atmospheric) — ammonia — phosphate (inorganic)

Legume-rhizobium symbiosis is of great biological and ecological significance. This interaction relies on the diffuse exchange of signal molecules between the two partners. The flavonoids secreted by host plant roots stimulate the expression of nodulation genes, which are essential for the synthesis and secretion lipochitooligosaccharides as Nod factors (NFs). On the other hand, recognition of NFs by LysM receptor-like kinase (LysM-RLKs)) produces a signaling cascade necessary for infection and nodular organ formation (Oldroyd, 2013). Nod factor synthesis requires the *nodABC* gene. NodC protein is a β-glucosaminyl transferase linking the UDP-N-acetyl glucosamine monomer. NodB removes the acetyl group from the chitin-like vertebral tip, while NodA stimulates transfection. Transfer of lipid acyl chains to the resulting free amino groups using acyl-ACP from fatty acid synthesis (Giraud et al., 2007; Hirsch et al., 2001).

2.6 *Bradyrhizobium* for peanut

A. hypogaea L. is frequently nodulated by slow-growing strains of rhizobacteria in the genus *Bradyrhizobium* (Bogino et al., 2006). There are many studies and differing opinions on the most effective agricultural strategies for peanut inoculation. There are many *Bradyrhizobium* inoculants trains used in the world such as C145, SEMIA6144, USDA4438, USDA 3456, SEMIA 6144, NC92, TAL1000, TH205, 32HI, (Kucey & Toomsan, 1988), CCBAU 51649, CCBAU 53363, CCBAU 10071, BR 3351, TAL173 and SUTN9-2 (Badawi et al., 2011; Kucey & Toomsan, 1988; Li et al., 2015; O'Hara et al., 1993; Taurian et al., 2013; Vicario et al., 2016; Yuttavanichakul et al., 2012). The efficiency in N_2-fixing of *Bradyrhizobium*, there are many reports mentioned to high efficiency in N_2-fixing for peanut growth promoting (Bell & Wright, 1994; Kucey & Toomsan, 1988). However, at the same time, the levels of N_2-fixation depend on efficiency of *Bradyrhizobium* stains (Florentino et al., 2010; Zazou et al., 2018). Inoculated peanuts received an average of 71 kg N/ha from immobilization; Un-uninoculated peanuts received an average of 62 kg N/ha (Kucey & Toomsan, 1988). The inoculation of certain rhizobial strains resulted in increased yield of seeds with high protein content (Vicario et al., 2016).

In peanuts, the infection occurs when the rhizobia from the root tissue directly colonize the subepidermal root tissue (the cortex) by crack entry into the lateral roots in an intercellular manner without the formation of infection threads in Fig.2.2, 2.3 and 2.4 (b) (Boogerd & van Rossum, 1997; Fabre et al., 2015; Raul et al., 2022; J. Sprent & James, 2007). In previous molecular studies on *AhCCamK* and *AhHK1* showed that *A. hypogaea* share a common SYM pathway for nodular development. This is because both the common symbiosis (SYM) and cytokinin pathways induce *AhCYCLOPS* expression in the initiation and coordinating nodule (crack-entry) in *A. hypogaea* (Das et al., 2019; Kundu & DasGupta, 2018; Sinharoy et al., 2009). Moreover, other studies identified the important role of cytokinins (CKs) as the main regulators of IT formation and nodule organogenesis (Gonzalez-Rizzo et al., 2006; Held et al., 2014; Jardinaud et al., 2016; Tirichine et al., 2007). Comparative analyzes of peanuts and other legumes showed the predominance of CKs and ethylene signaling pathways during nodule formation, the component CKs receptor and histidine kinase (HK1) plays an important role in the formation of nodular organs in

both peanuts and other legumes, in during nodules development *AhHK1*, *LjHK1* (*LHK1*) and *MtCRE1* had similar expression patterns. (Gonzalez-Rizzo et al., 2006; Kundu & DasGupta, 2018; Murray et al., 2007). The interaction between legumes and rhizobia activates SYM-pathway that recruits CK signaling for nodule primordia induction in the cortex by modulating the expression of auxin transporters (Frugier et al., 2008; Ng et al., 2015; Plet et al., 2011). SYM pathway and phytohormonal signaling together reprogram cortex cells and regulate their division, the nodule primordium is formed for the endocytic accommodation of symbionts (Mathesius et al., 2000; Suzaki et al., 2012), Most recently, it has revealed the role of CK signaling in peanuts and provides insights into the CK receptor silencing (RNAi) hypothesis, histidine Kinase1 (AhHK1), resulting in reduced nodules which indicates that the CK signal is mediated through this receptor is important for the initiation of the nodule primordium (Kundu & DasGupta, 2018).

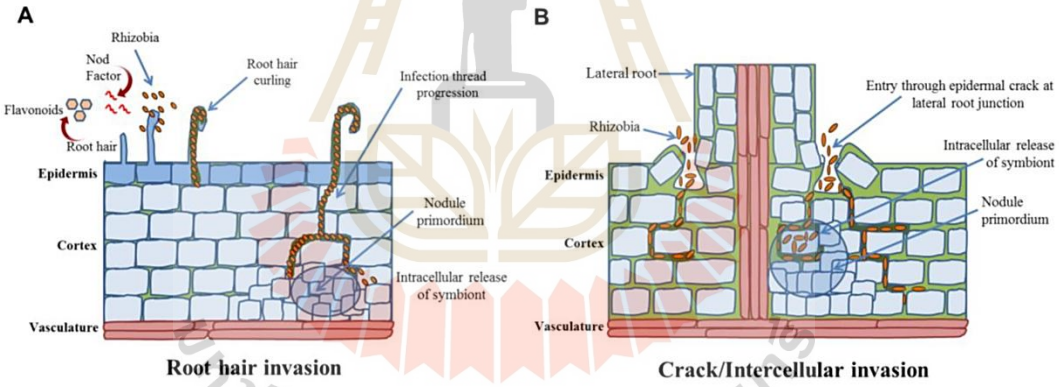

Figure 2.2 Rhizobial can enter and occupy the nodule in plant root by root hairs or cracks epidermis. (A) Root hair follicle invasion occurs through rhizobial adhesion to the root hairs and follicle deformation. (B). Intercellular rupture/invasion gives the epidermis and rhizobia direct access to cortical cells (V. Sharma et al., 2020).

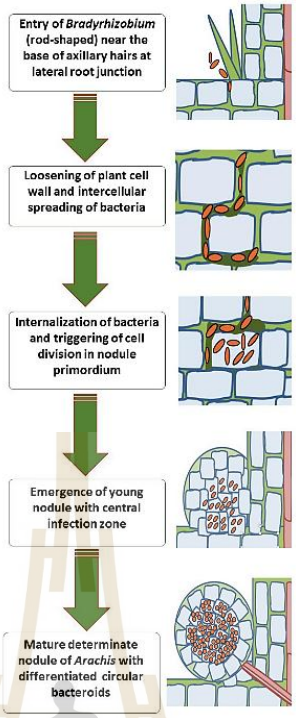

Figure 2.3 Continuous stages of nodule development in peanuts and invasion of *Bradyrhizobium* (crack-entry) (V. Sharma et al., 2020).

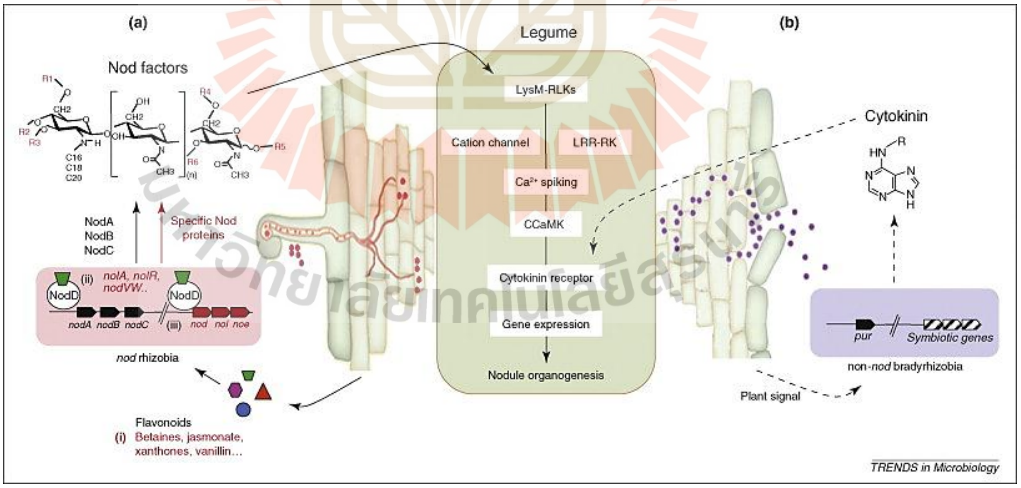

Figure 2.4 The nodulation strategies in rhizobia stimulate the formation of nodules on legumes by using a process infection thread (a) or crack-entry (b) (Masson-Boivin et al., 2009).

2.7 Competitiveness

Competitiveness is a very important practical factor because the broad use of seed culture has often proven unsuccessful as there are more competitive indigenous in the soil (Caldwell & Vest, 1970; Johnson et al., 1965). Therefore, the competition between the different rhizobia genotypes presents in the rhizosphere for occupying the root nodules should be intended. Rhizobia requires several steps to successfully occupy the nodule, such as adhesion to the root surface, inducing infection curling of the root hairs through the infection threads, chemical signal exchange with the host of the root to stimulate the development of the nodule, and differentiation in nitrogen-fixing bacteroid (Murray, 2011; Oldroyd et al., 2011; Suzaki & Kawaguchi, 2014; Wheatley & Poole, 2018). There is barrier in each step, so the competition for nodulation may be affected by these barriers. Thus, bacterial genes that act in the transition between steps or in the metabolic pathways associated with these processes were identified as determining the competitiveness of rhizobia (Althabegoiti et al., 2011; Ding et al., 2012; Frederix et al., 2014; Geddes et al., 2014; Jiménez-Zurdo et al., 1995; Lindström & Mousavi, 2020; Liu et al., 2017; Loh et al., 2002; Miller et al., 2007; Mongiardini et al., 2009; Okazaki et al., 2003; Patankar & González, 2009; Pobigaylo et al., 2008; Quelas et al., 2010, 2013, 2016; Sánchez et al., 2009; Soto et al., 1993; Triplett, 1988, 1990; Triplett & Barta, 1987; Van Dillewijn et al., 2001). In addition, the soil environment influences the competitiveness of some different genotypes, the acid soil pH favored the competition of slow-growing rhizobia over the fast-growing rhizobia, while the alkaline soil pH was the opposite (S. S. Yang et al., 2001). The high quality inoculant should contain rhizobial strains with high nitrogen fixation, highly competitive for nodulation and to a new to environmental conditions, in the environment many abiotic and biotic factors reduce the competitiveness and effectiveness of inoculant strain (Miljaković et al., 2022). Indigenous rhizobia are quantitative and qualitatively diverse but have low efficiency and often demonstrated higher competitiveness compared to highly effective inoculation strains (diCenzo et al., 2019), and horizontal gene transfer mechanisms that promote competition and survival without the need for symbiosis relationships (Provorov & Vorobyov, 2000). Furthermore, genetic, and physiological differences between inoculated and indigenous strains there seem to be a positional effect on the competitiveness of nodule occupancy, this is because indigenous strains are widely distributed according to soil characteristics, while the vaccinated strains remained

concentrated around the seeds (Bogino et al., 2008; López-García et al., 2002, 2009). Motility of inoculated rhizobia in the soil where the field capacity is generally low. Therefore, the distribution of rhizobia in the soil profile is important, this is because it allows the growing roots to come into contact with static rhizobia (López-García et al., 2002, 2009). Therefore, factors contributing to the generation of endophyte inoculated rhizobia need to be considered and the competitive outcomes of the indigenous populations should be considered, can increasing competitiveness for nodulation also improves the competitiveness of endophytes. The other side, the competitiveness of the leavening agent may be enhanced not only genetically, but also through the regulation of external factors such as the growth and preservation of bacteria the composition of culture media adding a stimulant and inoculation in the furrows instead of planting seeds.

2.8 *Bradyrhizobium* used as peanut inoculant in Thailand

A. strain TAL 173

Bradyrhizobium sp. stain TAL173 is commercial peanut bradyrhizobial strains recommend by the Department of Agriculture, Thailand for peanut farmer. This strain was isolated from several peanuts by isolated directly from 500 nodules. In the previous study, TAL 173 was co-inoculated with *Bacillus megaterium* and *B. subilis* that produce lytic protease enzyme and IAA to inhibit the root rot disease of peanut and increase nitrogen fixation in peanut (Yuttavanichakul et al., 2012)

B. strain SUTN9-2

Bradyrhizobium sp. strain SUTN9-2 was isolated from root nodules of *Aeschynomene americana* which originated in the rice fields of Thailand as a legume weed (Noisangiam et al., 2012). Moreover, this stain has ability to colonize in the intercellular space of rice tissue as endophyte bacteria and increase plant growth by increasing nitrogen fixation, producing IAA, and ACC deaminase production, all so found to symbiosis with mung bean (*Vigna radiata*) (Piromyou et al., 2015, 2017). In addition, this strain can nodulate on *A. hypogaea* and promote peanut growth (Noisangiam et al., 2012).

CHAPTER III

MATERIALS AND METHODS

3.1 To obtain bradyrhizobia from Lao PDR

3.1.1 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 3). The soil samples were collected at 20 samples/site with 1 kg/sample at a depth of 20 cm.

Figure 3.1 The provinces map of Lao PDR and sites of the soil sampling.

Table 3.1 The location of the soil sampling in Lao PDR.

No.	Place	Latitude and Longitude	Province
1	Sanphon village	18°09'04.4"N 101°17'06.9"E	Xayaboury
2	Sanphon village	18°09'04.4"N 101°17'06.9"E	Xayaboury
3	Sanphon village	18°08'54.6"N 101°17'18.0"E	Xayaboury
4	Sanphon village	18°08'54.6"N 101°17'18.0"E	Xayaboury
5	Sanphon village	18°08'51.3"N 101°17'16.9"E	Xayaboury
6	Sanphon village	18°08'54.6"N 101°17'18.0"E	Xayaboury
7	Sanphon village	18°08'54.6"N 101°17'18.0"E	Xayaboury
8	Sanphon village	18°08'54.6"N 101°17'18.0"E	Xayaboury
9	Sanphon range of hill	18°09'04.4"N 101°17'07.9"E	Xayaboury
10	Sanphon range of hill	18°09'04.4"N 101°17'07.9"E	Xayaboury
11	Sanphon range of hill	18°09'04.4"N 101°17'06.9"E	Xayaboury
12	Sanphon range of hill	18°09'04.4"N 101°17'07.9"E	Xayaboury
13	Sanphon range of hill	18°09'04.4"N 101°17'06.9"E	Xayaboury
14	Buamlao village	18°08'22.8"N 101°17'34.1"E	Xayaboury
15	Buamlao village	18°08'22.8"N 101°17'34.1"E	Xayaboury
16	Buamlao village	18°08'22.8"N 101°17'34.1"E	Xayaboury
17	Buamlao village	18°08'22.8"N 101°17'34.1"E	Xayaboury
18	Buamlao village	18°08'22.8"N 101°17'34.1"E	Xayaboury
19	Buamlao village	18°08'22.8"N 101°17'34.1"E	Xayaboury
20	Buamlao village	18°08'22.8"N 101°17'34.1"E	Xayaboury
21	Parkxarb village	18°7’36.22’’N 102°46’54.98’’E	Vientian capital
22	Parkxarb village	18°7’35.63’’N 102°47’34.7’’E	Vientian capital
23	Parkxarb village	18°7’22.97’’N 102°47’16.97’’E	Vientian capital
24	Parkxarb village	18°7’22.97’’N 102°47’16.97’’E	Vientian capital
25	Parkxarb village	18°7’36.23’’N 102°47’54.98’’E	Vientian capital

Table 3.1 The location of the soil sampling in Lao PDR (Con).

No.	Place	Latitude and Longitude	Province
26	Somsamai village	18°8’24.75’’N 102°49’23.55’’E	Vientian capital
27	Somsamai village	18°8’12.12’’N 102°48’56.17’’E	Vientian capital
28	Somsamai village	18°8’24.75’’N 102°49’23.55’’E	Vientian capital
29	Somsamai village	18°8’18.44’’N 102°49’9.86’’E	Vientian capital
30	Somsamai village	18°7’24.75’’N 102°49’23.55’’E	Vientian capital
31	Somsamai village	18°7’24.75’’N 102°49’23.97’’E	Vientian capital
32	Somsamai village	18°7’24.75’’N 102°49’16.97’’E	Vientian capital
33	Somsamai village	18°7’24.75’’N 102°49’23.87’’E	Vientian capital
34	Somsamai village	18°7’18.44’’N 102°49’98.86’’E	Vientian capital
35	Somsamai village	18°7’18.44’’N 102°49’9.86’’E	Vientian capital
36	Somsamai village	18°8’18.44’’N 102°49’9.86’’E	Vientian capital
37	Somsamai village	18°8’12.12’’N 102°48’56.17’’E	Vientian capital
38	Somsamai village	18°8’12.12’’N 102°48’56.17’’E	Vientian capital
39	Somsamai village	18°8’12.12’’N 102°48’56.17’’E	Vientian capital
40	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
41	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
42	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
43	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
44	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
45	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province

Table 3.1 The location of the soil sampling in Lao PDR (Con).

No.	Place	Latitude and Longitude	Province
46	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
47	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
48	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
49	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
50	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
51	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
52	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
53	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
54	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
55	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
56	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
57	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
58	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province

Table 3.1 The location of the soil sampling in Lao PDR (Con).

No.	Place	Latitude and Longitude	Province
59	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province
60	Aom village	18°57'48.1"N 103°17'47.4"E	Xaysomboon province

3.1.2 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 (Phommalath, 2019). 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 (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 minutes 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.

3.1.3 Exclusion of the redundant bradyrhizobial stains using BOX-PCR fingerprinting

3.1.3.1 DNA extraction

Each of bradyrhizobial isolate was inoculated into HMB (HEPES 1.3 g, MES 1.1 g, Yeast extract 0.25 g, L-Arabinose 1.0 g, Sodium-glutamate 1.0 g, Na_2SO_4 0.25 g, $CaCl_2.2H_2O$ 0.013 g, $FeCl_3$ 0.004 g, $MgSO_4.7H_2O$ 0.18 g, Na_2HPO_4 0.125 g, NaCl 0.3 g per distilled water 1 l with final pH 6.8 at 30°C for 7 days in shaking conditions for 200 rpm. After cultivation, 2 ml of bacterial cell culture were pelleted in 0.1% lauroylsarcosine in TEN buffers (0.1 M NaCl, 1mM EDTA pH 8, 10mM Tris-Cl pH 8) and centrifuged at 14,000 rpm for 5 minutes. Cells were resuspended in 200 µl of 20% sucrose in TEN buffer, 100 µl of 10% sodium dodecyl sulfate, 20 µl of lysozyme, 32 µl of RNase, and homogenized the tube by inversion prior to incubate at 37°C for 1 hour 30 minutes. There, 75 µl of 5M NaCl, and 500 µl of the mixture of saturated phenol, chloroform, and isoamyl alcohol (25:24:1) were added and centrifuged at 14,000 rpm for 5 minutes prior to transferred the supernatant to a new Eppendorf (repeat this step for 2-3 time). In addition, DNA pellets were precipitated by adding 0.5 volume of 3M NaOAC and 99% ethanol. Then, DNA pellets were washed using 70% ethanol. Finally, DNA pellets were dissolved in 30-50 µl of TE solution and added RNase 1:10 (v/v), DNA was observed by gel electrophoresis and stored at 4 °C. (Hartmann & Amarger, 1991) and purified DNA was used in other experiments.

3.1.3.2 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 minute at 95°C/1 cycle, followed by 30 denaturation cycles for 3 seconds at 94°C/35 cycles and 1 minute at 53°C/35 cycles, with annealing for 8 minutes at 65°C/35 cycles and elongation for 10 minutes at 69°C/1 cycle. The final elongation was at 4°C. The BOX-PCR products were visualized by 1.5% agarose gel electrophoresis for 45 minutes at 80V. The components of the PCR reaction are summarized in Table 3.2.

Table 3.2 Components of the BOX-PCR reaction.

Components	Final conc./25 µl	Stock conc.	Volumes
H_2O	-	-	15.75 µl
Dream tag buffer	1 X	10 X	2.5 µl
dNTPs	0.2 mM	10 mM	0.5 µl
Primer (BOX-A1R)	2 pmol	10 µM	5 µl
DreamTaq-polymerase (Purchase from Thermo Scientific, U.S.A)	1.25 unit	Varies	0.25 µl
DNA	-	-	1 µl

3.2 Acetylene reduction assay (ARA) for measuring nitrogenase activity

3.2.1 Plants growth conditions

The peanut seeds were surface sterilized as mentioned before. Leonard jars 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 culture was washed twice with 0.85% NaCl by centrifugation at 5,000 rpm for 10 minutes prior to measurement of the cell concentration at the optical density (OD) of 600 nm and being brought to OD600 of 1 in 0.85% NaCl. Each inoculant was inoculated into Leonard's jar 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 grown under lightroom conditions, at 25°C with a 16-h-day and 8-h-night cycle for 30 days.

3.2.2 Acetylene reduction assay (ARA)

Plants from each replication were taken from Leonard's jar. The adhering vermiculite was removed by gently shaking and washing Then, the root nodules was put in an 80 ml test tube that was closed with a rubber stopper. After that, the air was removed for an equivalent volume; acetylene gas was injected into these tubes to produce a final concentration of 10% per tube 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 C_2H_4 production (David et al., 1980).

3.3 Strain characterization

3.3.1 Physiological characteristics

3.3.1.1 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.

3.3.1.2 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 minutes at 4°C, and the supernatant was mixed with the Salkowski reagent (1:2) (Mohite, 2013). The mixtures were incubated for 15 minutes in darkness at 28±2°C and absorbance was measured at 530 nm. The produced IAA concentrations were estimated using a standard IAA curve.

3.3.1.3 Antibiotic resistance profile

The bradyrhizobial strains were first grown and washed, and the cell concentration was measured as previously described. 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.

Table 3.3 Type and concentration of antibiotic used in the study.

No.	Antibiotic	Final concentration (µg/ml)
1	Carbenicillin	500
2	Chloramphenicol	500
3	Erythromycin	250
4	Cefotaxime	20
5	Gentamicin	50
6	Tetracycline	100
7	Nalidixic acid	20; 50
8	Streptomycin	100; 200
9	Spectinomycin	100; 200
10	Kanamycin	50; 100
11	Ampicillin	50; 100

3.3.1.4 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 beef extract, 5 g peptone, and 1 l water) at 30°C for 7 days. Then, the cultures were centrifuged at 5,000 rpm for 10 minutes at 4°C, and the supernatant was mixed with CAS reagents (1:1). The mixtures were incubated for 20 minutes 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):

$$\text{Siderophore production PSU} = [(Ar - As)/Ar] \times 100$$

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)

3.3.2 Biochemical characteristics

The bradyrhizobial strains were first grown and washed, and the cell concentration measured, as previously mentioned. The APIZYM test and carbon utilization and nitrogen assimilation tests were used in this study.

3.3.2.1 APIZYM 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 examined based on color development along with the instructions in the manuscript.

3.3.2.2 Carbon utilization and nitrogen assimilation tests

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 buffered nodulation broth medium (BNM) (Ehrhardt et al., 1992) diet without succinate. Cell concentrations were measured at optical density (OD) at 600 nm and taken to OD600 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 broth (Ehrhardt et al., 1992). 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.

Table 3.4 Compounds of carbon sources and nitrogen sources for enzyme activity test of bradyrhizobia.

Growth Experiment	Compounds
Carbon Sources	Succinate [a], Malate [a], Malonate [a], Glucose [a], Sucrose [a], Phenol [b], Glutamate [a], Glutamine [a], Glycine [a], Alanine [a], Arginine [a], Asparagine [a], Proline [a], Tryptophan [b], Lysine [a], Sorbitol [a], Myoinositol [a], Arabinose [a], Mannitol [a]
Nitrogen Sources	NH_4Cl [c], KNO_3 [c], Urea [c], NH_4NO_3 [c]
Mixed C and N	Glucose [a] + NH_4Cl [c], Glucose [a] + KNO_3 [c], Glucose [a] + Urea [c], Glucose [a] + NH_4NO_3 [c], Succinate [a] + NH_4Cl [c], Succinate [a] + KNO_3 [c], Succinate [a] + Urea [c], Succinate [a] + H_4NO_3 [c]

[a] Carbon Sources were used for 20 mM

[b] Carbon Sources were used for 5 mM

[c] Nitrogen Sources were used for 10 mM

3.3.3 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 grown under lightroom conditions, at 25°C with a 16-h-day and 8-h-night cycle for 30 days.

Table 3.5 Plant variety and chemical concentrate for seeds sterilization.

No.	Plant	Sterilization
1	*Aeschynomene americana*	$H_2SO_4$for 30 minutes
2	*A. indica*	$H_2SO_4$for 30 minutes
3	*A. evenia*	$H_2SO_4$for 30 minutes
4	*A. afraspera*	$H_2SO_4$for 30 minutes
5	*Arachis hypogaea* (Thainan 9)	95% ethanol 20 second and NaOCl 30 second
6	*Cajanus cajan* (Linn.) Millsp.	95% ethanol 30 second and NaOCl 5 minutes
7	*Crotalaria juncea*	$H_2SO_4$for 30 minutes
8	*Glycine max* (Chiang Mai 60)	95% ethanol 20 second and NaOCl 30 second
9	*Indigofera tinctoria*	$H_2SO_4$for 30 minutes
10	*Lotus japonicus*	$H_2SO_4$for 30 minutes
11	*Macroptilium atropurpureum*	$H_2SO_4$for 30 minutes
12	*Psophocarpus tetragonolobus*	95% ethanol 30 second and NaOCl 5 minutes
13	*Samanea saman*	$H_2SO_4$for 30 minutes
14	*Sesbania rostrata*	$H_2SO_4$for 30 minutes
15	*Vigna radiata* (SUT1)	95% ethanol 30 second and NaOCl 5 minutes
16	*V. mungo* (U thong 2)	95% ethanol 30 second and NaOCl 5 minutes
17	*V. subterranean*	95% ethanol 30 second and NaOCl 5 minutes
18	*V. unguiculata ssp. Sesquipedalis*	95% ethanol 30 second and NaOCl 5 minutes

3.3.4 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 DNA containing the vectors was sequenced by A T G C Co. Ltd., South Korea.

The 16S rRNA, *recA*, *atpD*, *glnII*, and *rpoB* sequences were analyzed and confirmed in the 5′ and 3′with primer sequences, and the phylogenetic tree was constructed 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.

Table 3.6 Primer sequences and reference.

Gene	Primer	Sequence (5`- 3`)	Reference
recA	TSrecA. F	CAACTGCMYTGCGTATCGTCGAAGG	(Stepkowski et al., 2005)
	TSrecA. R	CGGATCTGGTTGATGAAGATCACCATG	
atpD	TSatpD. F	TCTGGTCCGYGGCCAGGAAG	(Stepkowski et al., 2005)
	TSatpD. R	CGACACTTCCGARCCSGCCTG	
glnII	TSglnII. F	AAGCTCGAGTACATCTGGCTCGACGG	(Stepkowski et al., 2005)
	TSglnII, R	SGAGCCGTTCCAGTCGGTGTCG	
ropB	rpoB83. F	CCTSATCGAGGTTCACAGAAGGC	(Martens et al., 2008)
	rpoB1061. R	AGCGTGTTGCGGATATAGGCG	
16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	(Weisburg et al., 1991)
	rP2	CTTAAGGAGGTGATCCAGCC	

3.3.5 Competitiveness of selected peanut bradyrhizobial strains

3.3.5.1 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 (1×106 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 and the plants were grown under lightroom conditions at 25°C 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.).

3.3.5.2 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 and *Escherichia coli* strain HB101 containing pRK2013 plasmid (Figureureurski 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 on HM agar plates, using pRK2013 as a helper plasmid. Transconjugants were selected on HM agar plates containing 200 µg/ml streptomycin and 50 µg/ml nalidixic acid (Okubo et al., 2013; Piromyou et al., 2015a).

In this experiment, soil was collected from the campus of the Faculty of Agriculture of the National University of Lao PDR, 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 (OD600=1, 1 × 109 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 (3.3.5.1).

3.4 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.

CHAPTER IV
RESULTS AND DISCUSSIONS

4.1 Isolation bradyrhizobia from soil and strain selection

To obtain bradyrhizobia nodulating peanuts from Lao PDR, the soil samples were collected from 3 provinces in the Lao PDR including Xayaboury and Xaysomboon provinces and Vientiane capital, and BOX-PCR fingerprinting was used to exclude the same strains. This was because BOX-PCR is an effective method for detecting heterogeneity between species (Menna et al., 2009). The 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. 4.1).

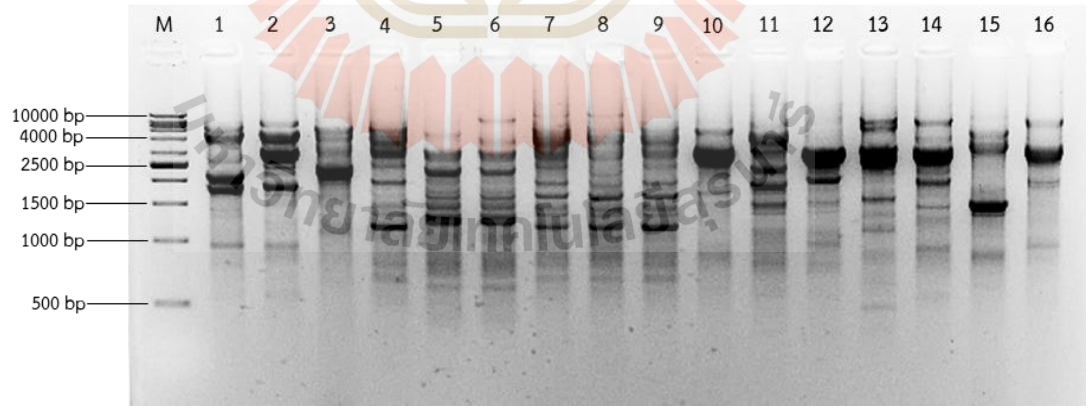

Figure 4.1 Agarose gel electrophoresis of BOX-PCR fingerprinting patterns of isolates from nodules. (M) Marker, (1) TAL173, (2) SUTN9-2, (3) SPXBL-07, (4) SPXBL-06, (5) BLXBL-03, (6) BLXBL-02, (7) SPXBL-05, (8) SPXBL-04, (9) SPXBL-03, (10) PMVTL-02, (11) PMVTL-01, (12) SMVTL-02, (13) SMVTL-01, (14) SPXBL-02, (15) SPXBL-01 and (16) BLXBL-01.

4.2 The selection of bradyrhizobia

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. 4.2). The highest nitrogenase activity was derived from isolate PMVTL-01. The isolates SPXBL-02, SPXBL-03, SPXBL-06, BLXBL-03, PMVTL-02, and SMVTL-02 were higher than TAL173 but not significantly 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 when compared to TAL173 (Fig. 4.2).

The nodule number performed the highest by SUTN9-2. 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. 4.3).

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.

Normally, the nodules of peanut plant is smaller with higher nitrogen-fixing activity than soybean, the nodules size and nodules number have an effect on the nitrogen-fixing activity (Tajima et al., 2007). There are strains of *Bradyrhizobium* sp. able to nodulate on peanuts, which showed a high correlation between nitrogen activity, shoot weight, nodule number and nodule dry weight (Li et al., 2015, 2019; Nigam et al., 1985; Wynne et al., 1983). Therefore, the selection of the proper rhizobial strains may result in improvement in plant phenotype, shoot weight, nodule number and nodule dry weight (Wynne et al., 1983).

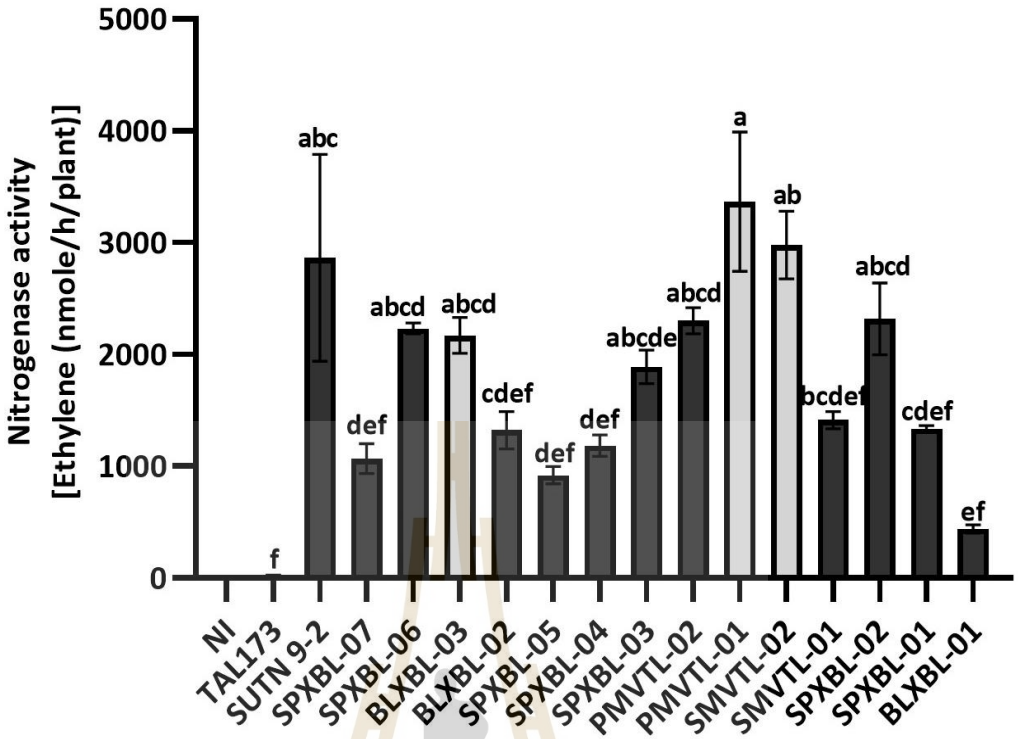

Figure 4.2 Nitrogenase activity of nodules measured by acetylene reduction assay. The results were measured in peanut under lightroom conditions at 30 DAI, non-inoculated (NI). Values with different letters in each treatment differed significantly at $P \geq 0.05$ (n = 3).

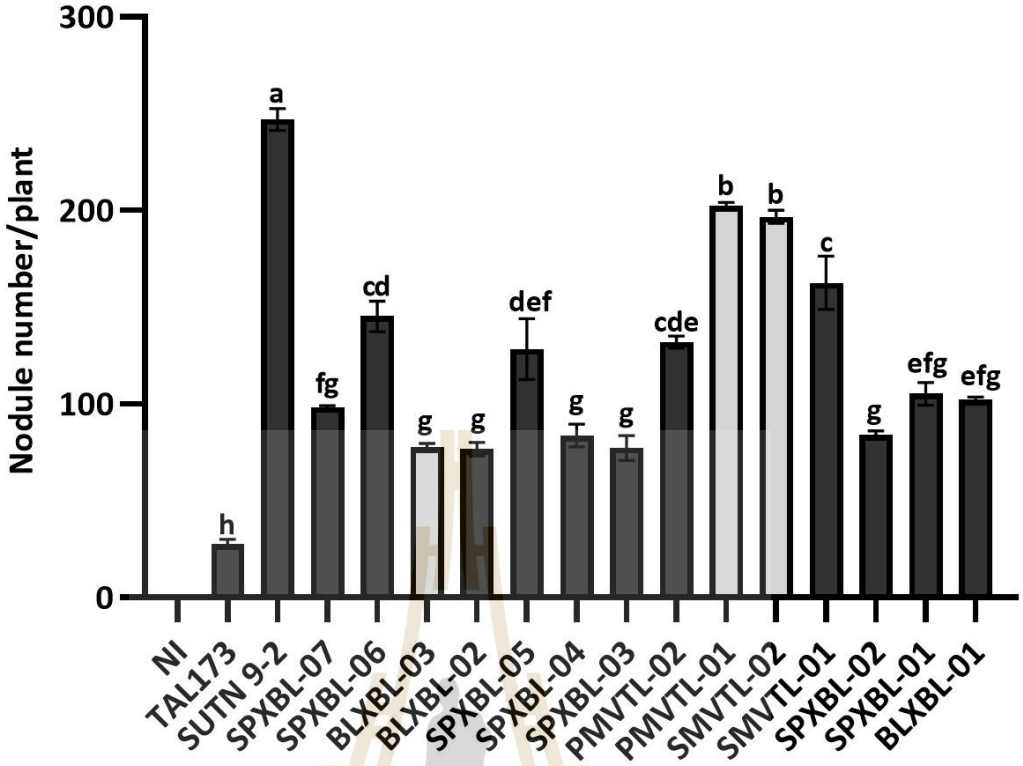

Figure 4.3 Nodule number was measured in peanut under lightroom conditions at 30 DAI, non-inoculated (NI). Values with different letters in each treatment differed significantly at $P \geq 0.05$ (n = 3).

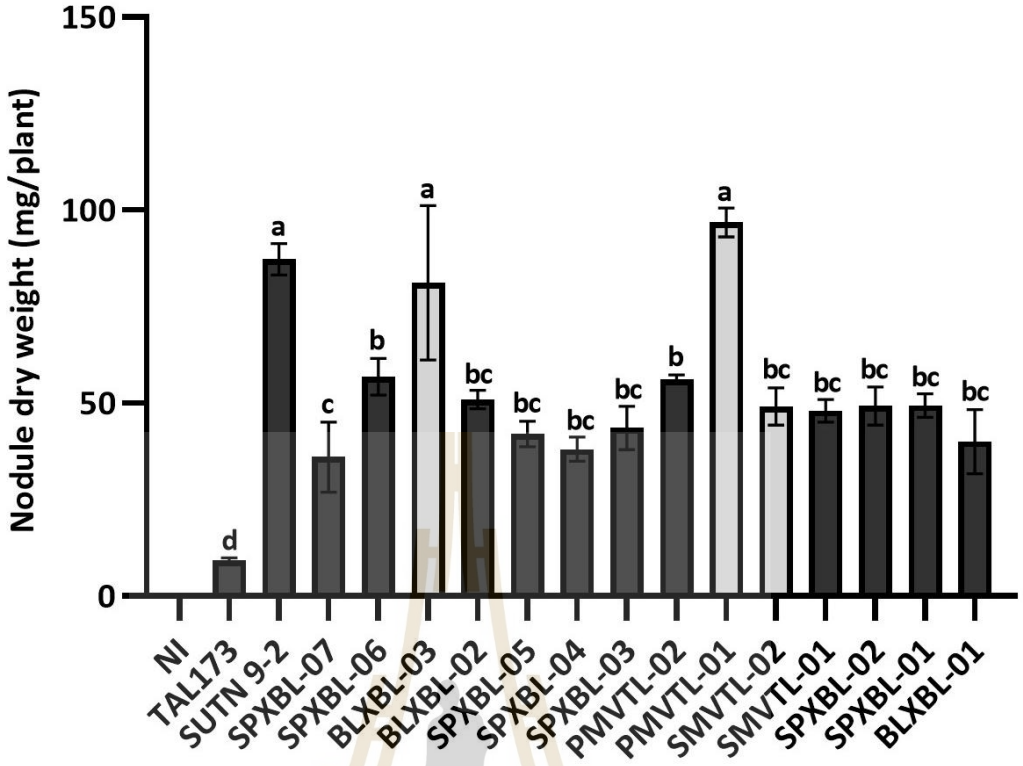

Figure 4.4 Nodule dry weight was measured in peanut under lightroom conditions at 30 DAI, non-inoculated (NI). Values with different letters in each treatment differed significantly at $P \geq 0.05$ (n = 3).

4.3 Physiological characteristics

4.3.1 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.4.5). The rhizobium isolates were tested on YEMA plates containing bromothymol blue (BTB) which indicated that fast-growing or slow-growing isolates (Saeki et al., 2005; M. P. Sharma et al., 2010). All most 3 isolates were were found as slow-growing based on YEMA containing BTB test. The rhizobium isolates were shown the same patten when compared with reference strains in which produced yellow colour due to acid production and produced bule colour due to alkaline production in fast and slow growing strains, respectively (Hungria et al., 2001; Saeki et al., 2005). 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. 4.5A). The SMVTL-02 showed a

bigger size than PMVTL-01 and BLXBL-03; the colony was translucent, convex, and slime (Fig. 4.5B). 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. 4.5C).

The results of IAA produced from selected isolates demonstrated that BLXBL-03 showed a dark pink color as compared to the other strains (Fig. 4.6A) 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, which was 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. 4.6B). IAA is a plant phytohormone of the auxin family, an important responsibility for regulating plant physiological processes, including light response, enlargement, cell division and tissue differentiation (Shokri & Emtiazi, 2010; Teale et al., 2006). In the various reports exist about the synthesize of phytohormones by rhizobial bacteria, *Asosperilum*, *Azotobacter*, fungi and algae are producers of auxins (Farzana et al., 2007; Ma et al., 2002; Shokri & Emtiazi, 2010; van Loon & Bakker, 2003). The level of IAA production depended on rhizobium isolates (Mandal et al., 2009), and IAA is also an autoregulatory factor for root nodulation (Asim et al., 2013).

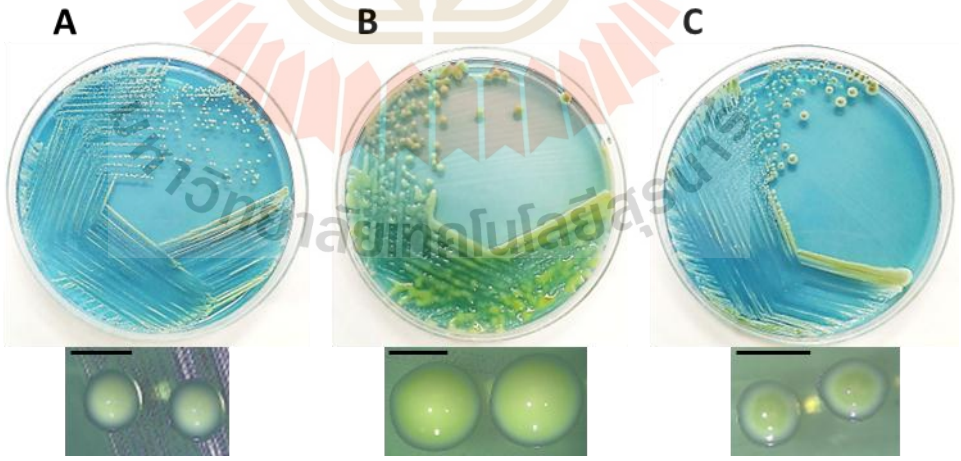

Figure 4.5 Production of acid-alkaline by *Bradyrhizobium* sp. strains at 7 days. (A) PMVTL-01, (B) SMVTL-02 and (C) BLXBL-03. The colonies were observed under Leica EZ4 with moticam X3 (Scale bars 300 µm).

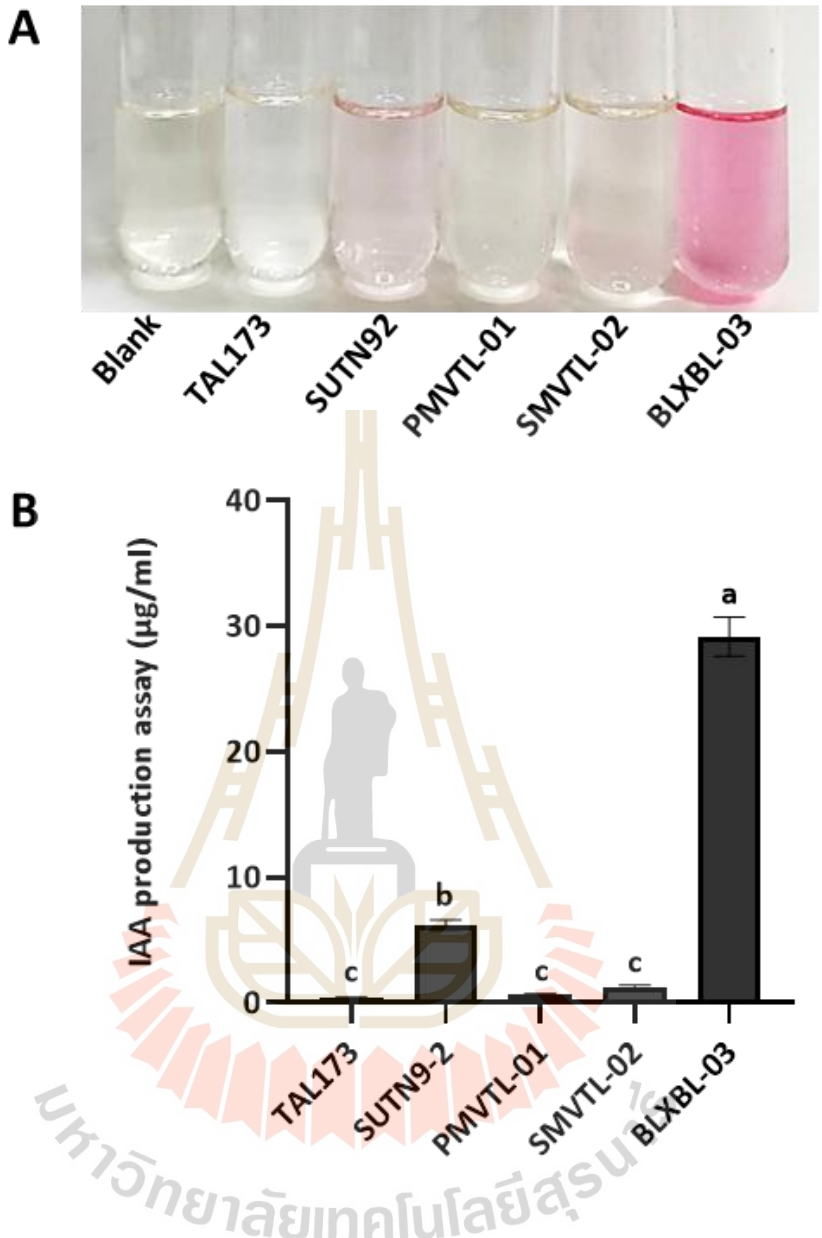

Figure 4.6 Production of indole-3-acetic acid (IAA) by *Bradyrhizobium* sp. strains (7 days). (A) Control was culture medium without bacterial inoculation mixed with Salkowski reagent, color patterns were observed absorbance at 530 nm, (B) Quantitative evaluation of IAA production by *Bradyrhizobium* sp. strains. Values with different letters in each treatment differed significantly at $P \geq 0.05$ (n = 3).

4.3.2 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^{-1}) 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^{-1}), and ampicillin (100 μg ml^{-1}). On the other hand, this strain showed spontaneity to erythromycin, streptomycin, spectinomycin, and kanamycin (50 μg ml^{-1}). 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 4.1).

Bradyrhizobium sp. isolated from peanuts was shown to be antibiotic-resistant to carbenicillin, nalidixic acid, and spectinomycin but some isolates showed sensitivity 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; Teamtisong, 2000). 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; Teamtisong, 2000; Young and Chao, 1989).

Table 4.1 *Bradyrhizobium* sp. strains and their intrinsic antibiotic profiles. Resistant (R), Sensitive (S) and Spontaneous (SP).

No.	Antibiotic	Concentration (µg)	Strains		
			PMVTL-01	SMVTL-02	BLXBL-03
1	Carbenicillin	500	R*	R	R
2	Chloramphenicol	500	S	S	S
3	Erythromycin	250	SP	SP	SP
4	Cefotaxime	20	R	S	SP
5	Gentamicin	50	S	S	R
6	Tetracycline	100	S	S	S
7	Nalidixic acid	20	R	R	R
		50	SP	R	R
8	Streptomycin	100	SP	SP	SP
		200	SP	SP	SP
9	Spectinomycin	100	SP	SP	SP
		200	SP	SP	SP
10	Kanamycin	50	S	SP	SP
		100	S	S	SP
11	Ampicillin	50	R	S	R
		100	R	S	R

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

4.3.3 Determination of siderophore production

The results of siderophore production indicated that different *Bradyrhizobium* sp. strains showed variation (Fig. 4.7A and 4.7B). SMVTL-02 was the most proficient siderophore producer when compared to other strains (Fig. 4.7B). 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. 4.7B). Meanwhile, BLXBL-03 showed significantly lower than every strain (Fig. 4.7B).

Bacteria need iron to survive, grow, and developed a variety of mechanisms for the solubility of Fe^{3+} (Guerinot, 1994). Many species of rhizobia belonging to produce siderophores 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 by 200 fold (Carson et al., 1992). In this study indicate that SMVTL-02 is the highest siderophore producer, while the lowest is BLXBL-03 (Fig. 4.7B). 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 (Bar-Ness et al., 1991; Igiehon et al., 2019; Igiehon and Babalola, 2018; Tkacz and Poole, 2015). 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).

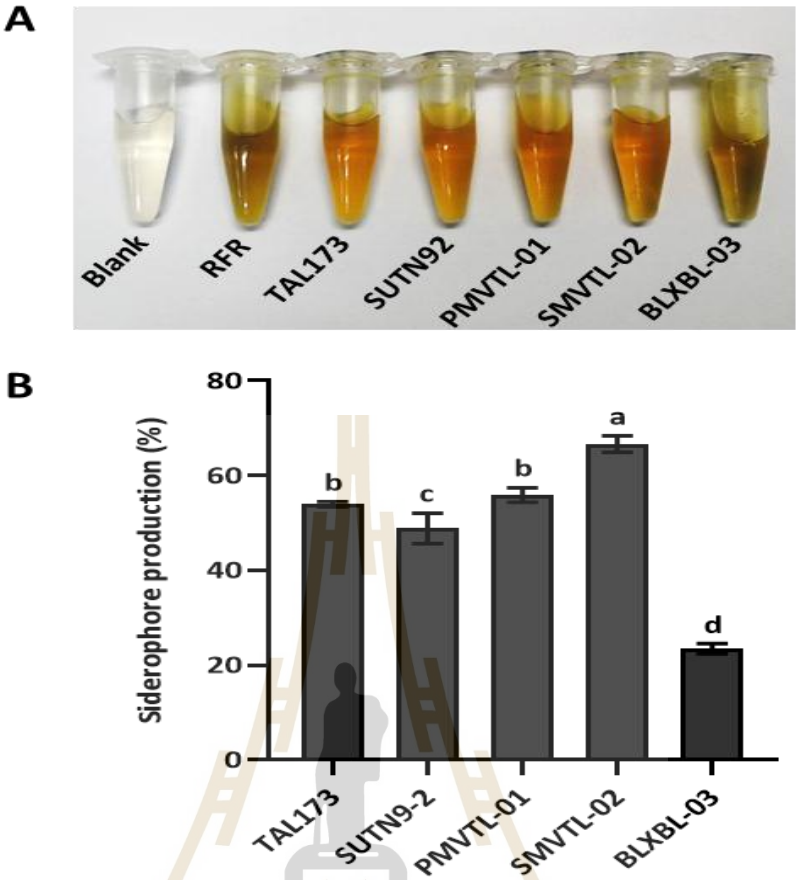

Figure 4.7 Siderophore production of by *Bradyrhizobium* sp. strains (7 days). (A) Reference was culture medium without bacterial inoculation with CAS reagent (RFR). The color changes were observed absorbance at 630 nm. (B) Quantitative analysis of siderophore production of by *Bradyrhizobium* sp. strains. Values with different letters in each treatment differed significantly at $P \geq 0.05$ (n = 3).

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

The result of the APIXYM test demonstrated that enzyme activity derived from most of the strains 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 potassium nitrate (reduction of nitrates to nitrites), gelatin (bovine origin), and 4-Nitrophenyl-βD-galactopyranoside (Table 4.2). 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, negative results were found in L-tryptophan, N-acetyl glucosamine, D-maltose, potassium, gluconate, capric acid, adipic acid, and malic acid. PMVTL-01, SMVTL-02, and BLXBL-03 showed positive results in D-glucose and L-arabinose, while they showed negative results in L-tryptophan, D-mannose, D-mannitol, N-acetyl-glucosamine, D-maltose, potassium, gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid.

Most strains could grow on both anaerobic ($-O_2$) and microaerobic aerobic ($+O_2$) conditions with succinate, glutamine, asparagine, proline, tryptophan, and sorbitol (Table 4.3). The most of strains could grow on both anaerobic ($-O_2$) and microaerobic aerobic ($+O_2$) condition with succinate, glutamine, asparagine, proline, tryptophan, and sorbitol. 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.

From, the results of the APIZYM test and the utilization of various carbon and nitrogen sources analyses showed 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 76T, *B. liaoningense* USDA 3622T, *B. yuanmingense* CCBAU 10071T, *B. diazoefficiens* USDA 110T, and *B. japonicum* USDA 6T were grown on glucose (Wójcik et al., 2019).

Table 4.2 Result of hydrolytic enzymes activity assayed using API ZYM 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	-	-	-	-	-

Table 4.2 Result of hydrolytic enzymes activity assayed using API ZYM kit (Con),

Active ingredients	Strains				
	TAL173	SUTN9-2	PMVTL-01	SMVTL-02	BLXBL-03
D-Maltose	**-**	**-**	**-**	**-**	-
Potassium	-	-	-	-	-
Gluconate	-	-	-	-	-
Capric acid	-	-	-	-	-
Adipic acid	-	-	-	-	-
Malic acid	-	-	-	-	-
Trisodium citrate	+	+	-	-	-
Phenylacetic acid	+	+	-	-	-

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

Table 4.3 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.

4.4 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 4.4). All of the isolates were not nodulated on *A. indica*, *A. evenia*, *Glycine max* (Chiang Mai 60), *Lotus japonicus*, *Psophocarpus tetragonolobus*, *Samanea saman*, and *Sesbania rostrata*. In addition, only isolate SMVTL-02 was not nodulated on *Cajanus cajan* (Linn.) Millsp. and only isolate BLXBL-03 was not nodulated on *Crotalaria juncea* and *Vigna radiata* (SUT1).

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 *A. americana* (Noisangiam et al., 2012; Nzoué et al., 2009), *A. afraspera* (Bonaldi et al., 2011), *Arachis hypogaea* (Thainan 9) (Songwattana et al., 2017), *C. cajan (Linn.) Millsp.* (Alaswad et al., 2019), *C. juncea* (You et al., 2002), *I. tinctoria* (Leelahawonge et al., 2010), *M. atropurpureum* (Yuhashi et al., 2000), *V. 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).

4.5 Acetylene reduction assay

The nitrogenase activity of *Bradyrhizobium* sp. strains PMVTL-01, SMVTL-02, and BLXBL-03 compared to the commercial strain including *Bradyrhizobium* sp. strains TAL173 and SUTN9-2 was confirmed (Fig. 4.8). From the results of ARA, SMVTL-02 performed highest nitrogenase activity (Fig. 4.8B). PMVTL-01 showed significantly nitrogenase activity higher than BLXBL-03 and TAL173 but was not significant when compared to SUTN9-2 (Fig. 4.8B). BLXBL-03 performed significantly in terms of lower nitrogenase activity than PMVTL-01, SMVTL-02, and SUTN9-2 but higher than TAL173 (Fig. 4.8B).

For the nodule numbers, the higher numbers were found in SMVTL-02 and SUTN9-2 (Fig. 4.9). PMVTL-01 showed a reduction in nodule numbers, while

performed nitrogenase activity higher nodules number than BLXBL-03 and TAL173 (Fig. 4.9). BLXBL-03 showed a significantly lower nodule number than every strain except TAL173 (Fig. 4.9). The nodule dry weight and total plant dry weight showed nitrogenase activity higher and not significantly in PMVTL-01, SMVTL-02, and SUTN9-2 (Fig. 4.10, 4.11), 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. 4.10, 4.11).

Table 4.4 The result of *Bradyrhizobium* sp. nodulation in 18 kinds of legume.

No.	Plant	Strains		
		PMVTL-01	SMVTL-02	BLXBL-03
1	*Aeschynomene americana*	+*	+	+
2	*A. indica*	-	-	-
3	*A. evenia*	-	-	-
4	*A. afraspera*	+	+	+
5	*Arachis hypogaea* (Thainan 9)	+	+	+
6	*Cajanus cajan* (Linn.) Millsp.	+	-	+
7	*Crotalaria juncea*	+	+	-
8	*Glycine max* (Chiang Mai 60)	-	-	-
9	*Indigofera tinctoria*	+	+	+
10	*Lotus japonicus*	-	-	-
11	*Macroptilium atropurpureum*	+	+	+
12	*Psophocarpus tetragonolobus*	-	-	-
13	*Samanea saman*	-	-	-
14	*Sesbania rostrata*	-	-	-
15	*Vigna radiata* (SUT1)	+	+	-
16	*V. mungo* (U thong 2)	+	+	+
17	*V. subterranean*	+	+	+
18	*V. unguiculata* ssp. *Sesquipedalis*	+	+	+

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

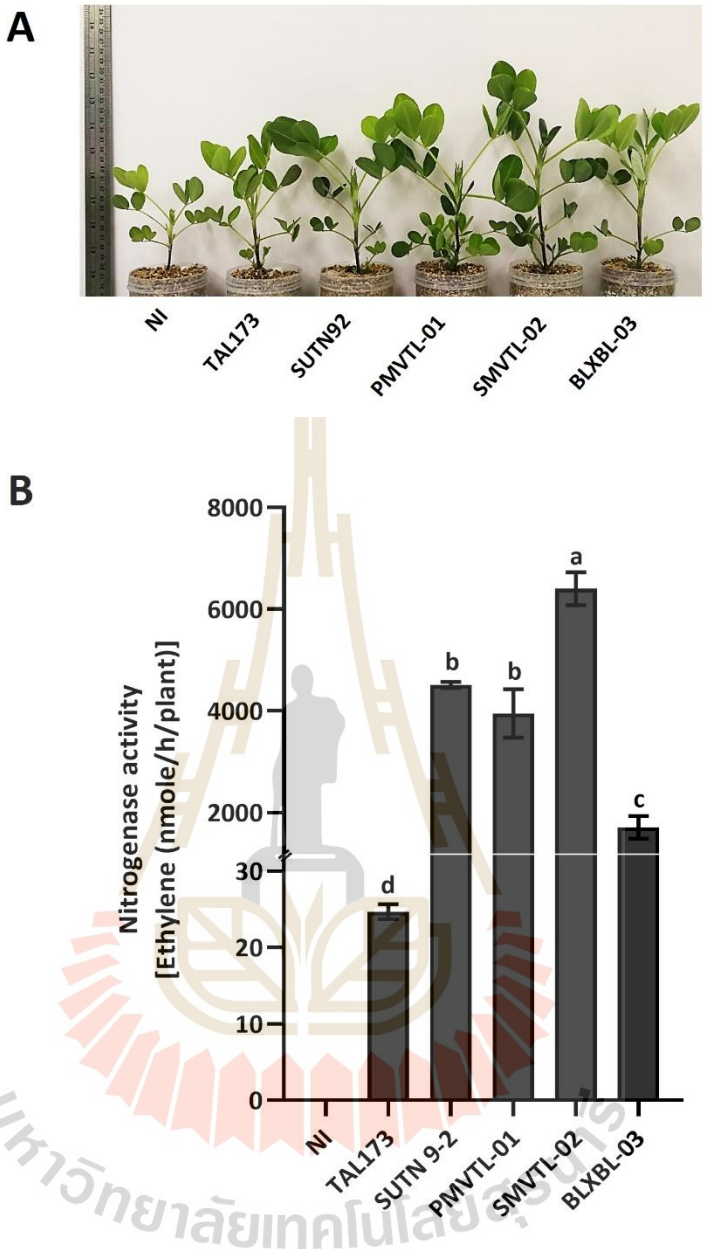

Fig 4.8 Peanut growth promotion and symbiotic properties. (A) phenotype of peanut when inoculated with *Bradyrhizobium* sp., (B) nitrogenase activity measured by acetylene reduction assay (ARA). The results were measured in peanut under lightroom conditions at 30 DAI, non-inoculated (NI). Values with different letters in each treatment differed significantly at $P \geq 0.05$ (n = 3).

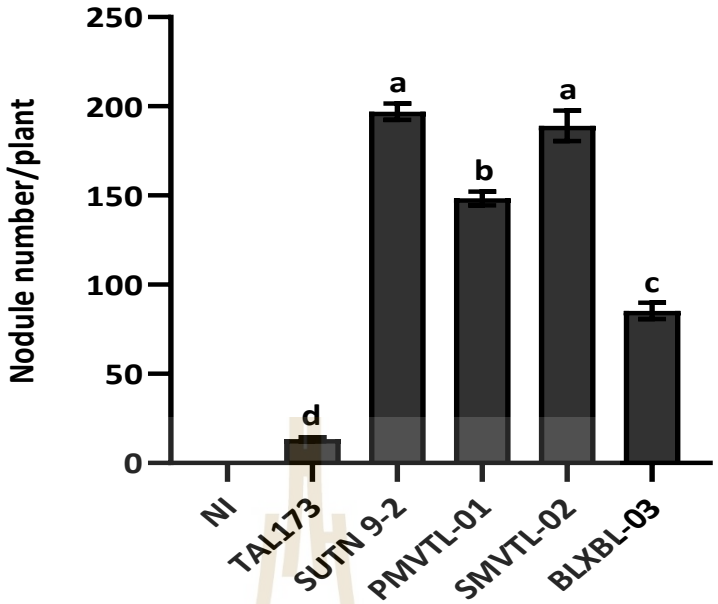

Fig 4.9 Nodule number, the results were measured in peanut under lightroom conditions at 30 DAI, non-inoculated (NI). Values with different letters in each treatment differed significantly at $P \geq 0.05$ (n = 3).

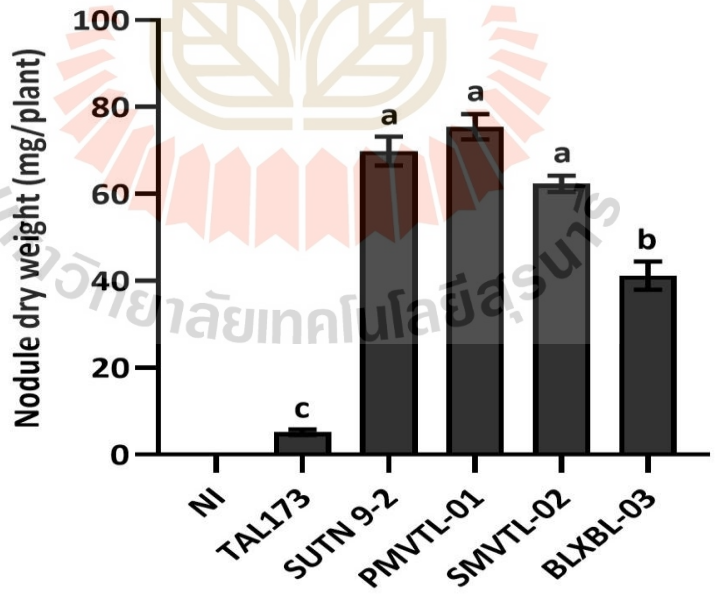

Fig 4.10 Nodules dry weight, the results were measured in peanut under lightroom conditions at 30 DAI, non-inoculated (NI). Values with different letters in each treatment differed significantly at $P \geq 0.05$ (n = 3).

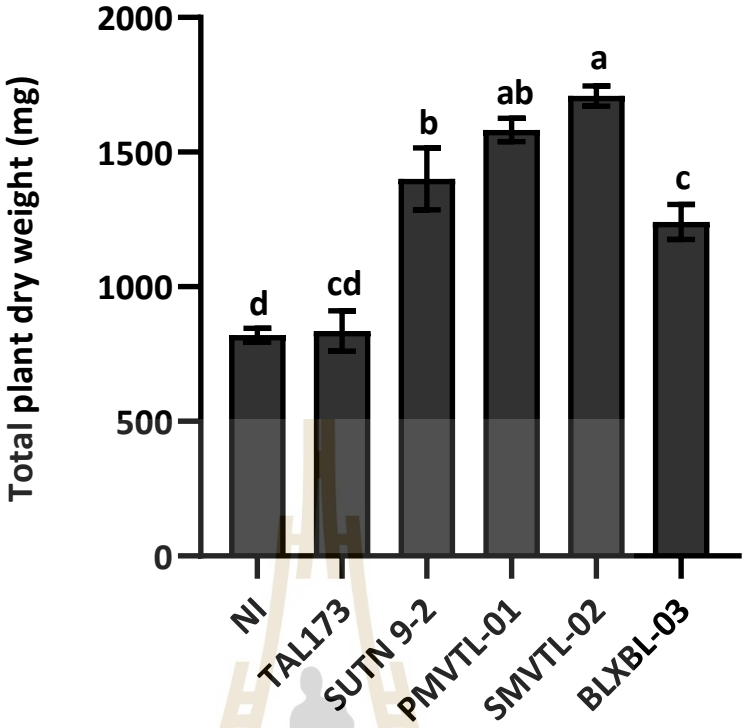

Fig 4.11 Total plant dry weight, the results were measured in peanut under lightroom conditions at 30 DAI, non-inoculated (NI). Values with different letters in each treatment differed significantly at $P \geq 0.05$ ($n = 3$).

4.6 Genotypic characteristics of isolates PMVTL-01, SMVTL-02 and BLXBL-03

4.6.1 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. 4.12). These results suggested that from the 16S rRNA gene (1,444 bp), PMVTL-01 and SMVTL-02 showed the highest similarity with 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 a final bootstrap support of 90%.

4.6.2 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. 4.13-4.17).

In the *recA* gene; PMVTL-01 and SMVTL-02 were changed the position closer to *B. liaoningense* LMG18230 and another cluster followed by the first cluster, including *Bradyrhizobium* sp. DOA9, *B. yuanmingense* LMG21827, *B. yuanmingense* LMG21827, and the second cluster including *Bradyrhizobium* sp. ORS3257, *Bradyrhizobium* genosp. SA-3 str. Rp7b and *B. zhanjiangense* CCBAU51781. While BLXBL-03 was clustered with *Bradyrhizobium* sp. CCBAU51745 with a bootstrap support of 99%, followed by the *B. manausense* BR3351 at 51% (Fig. 4.13).

The tree built with the *atpD* gene; PMVTL-01 and SMVTL-02 were clustered in *Bradyrhizobium* sp. SUTN9-2 and *B. yuanmingense* LMG21827 with a bootstrap support of 94%, for BLXBL-03 was closer in two subgroups, the first clustering four 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. 4.14).

The tree built with the *glnII* gene also resulted of PMVTL-01 and SMVTL-02 were divided in two subgroups: the first clustering four strains with higher similarity with *Bradyrhizobium* genosp. SA-4 str. CB756 with a bootstrap support of 98% and the second with two strains grouping with *B. arachidis* 62303 and *B. arachidis* LMG26795 with a bootstrap support of 82% (Fig. 4.15).

A greater variance was also found with the *rpoB* gene; PMVTL-01 and BLXBL-03 were with high similarity to *Bradyrhizobium* sp. ORS3257. Meanwhile, PMVTL-01 was closely to *Bradyrhizobium* sp. CCBAU51745 and *B. manausense* BR3351 with a bootstrap support of 98% (Fig. 4.16).

From the analysis of the 16S rRNA (Fig. 4.12) and the combination of 5 genes (Fig. 4.17), the section of *Bradyrhizobium* in two main groups was clear, the position as the same of 16S rRNA gens tree. PMVTL-01 and SMVTL-02 were closer to *B. zhanjiangense* CCBAU51781 and *Bradyrhizobium* genosp. SA-3 str. Rp7b with a 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 a bootstrap support of 99% and the second subgroup including *B. guangdongense* CCBAU51658, *B. guangdongense* CGMCC1.15034, and *B. guangzhouense* CCBAU53424 with a bootstrap support of 95%.

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, CGMCC1.15034, and *B. guangzhouense* CCBAU53424. All the closely related strains in this phylogenetic tree were isolated from *A. hypogaea* (Li et al., 2021, 2019, 2015), except *B. manausense* BR3351, which was isolated from V. unguiculata (Silva et al., 2014).

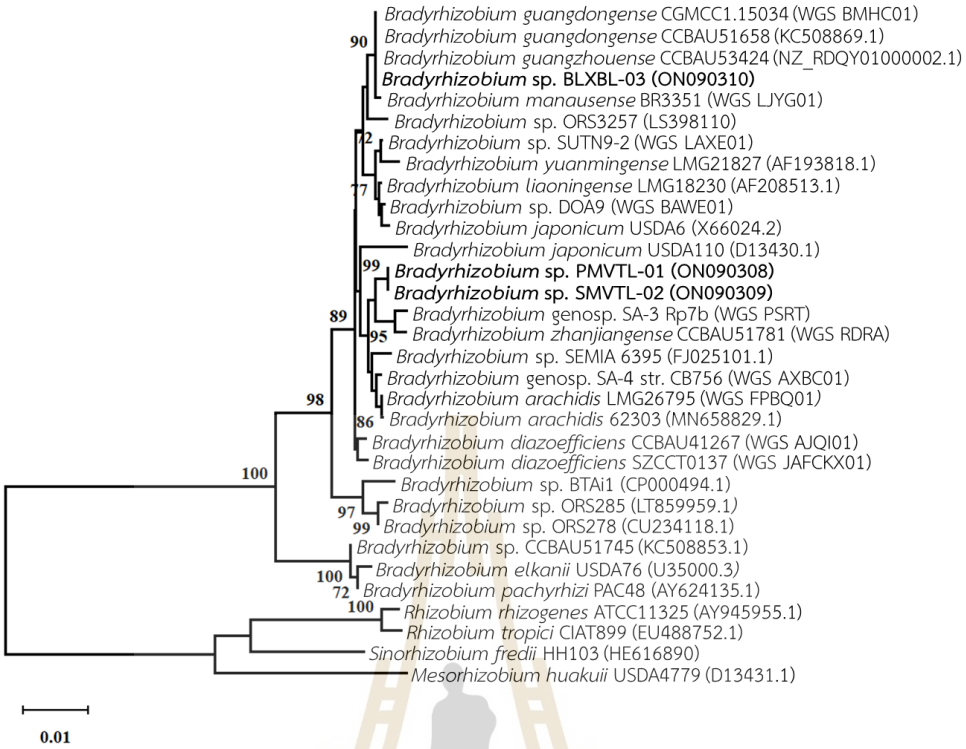

Fig 4.12 16S rRNA phylogenetic tree, phylogenetic tree of 16S rRNA sequences acquired by the neighbor-joining method appearing the phylogenetic relationship of the *Bradyrhizobium* sp. with the correlated species. The phylogenetic tree procreated based on 16S rRNA genes of the 29 rhizobial strains. Neighbor-joining 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.

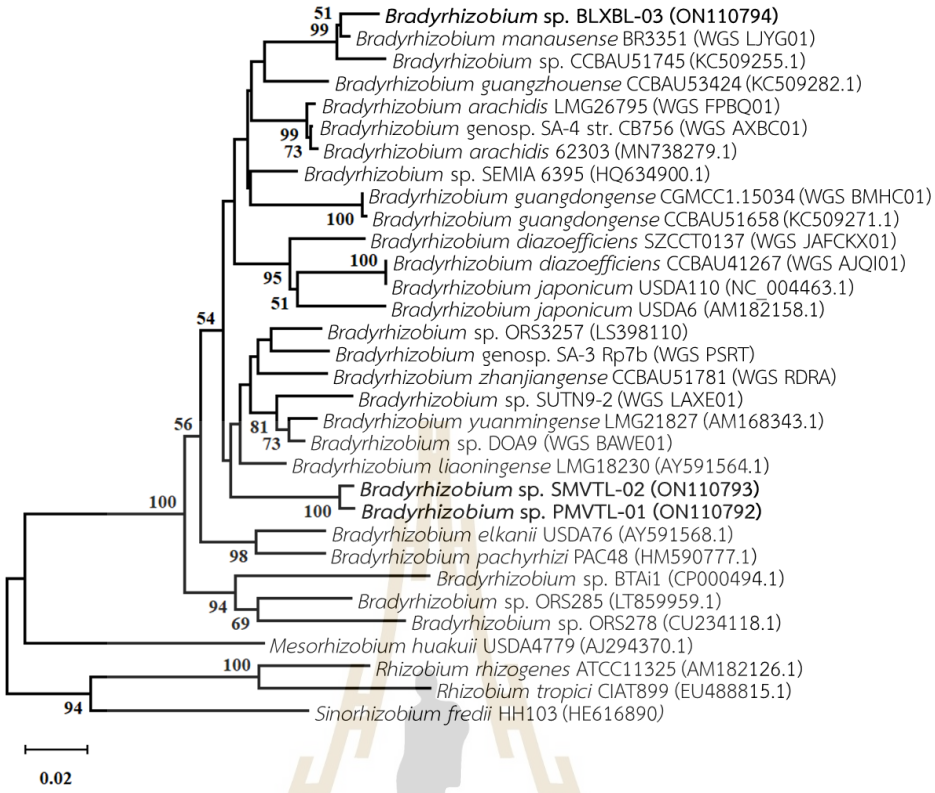

Fig 4.13 *recA* gene phylogenetic tree, the phylogenetic tree procreated based on housekeeping genes were conducted in MEGA11, the bootstrap is calculated inferred from 1000 replicates and shown at the respective nodes for values >50%, the scale bar represents 0.02% sequence divergence.

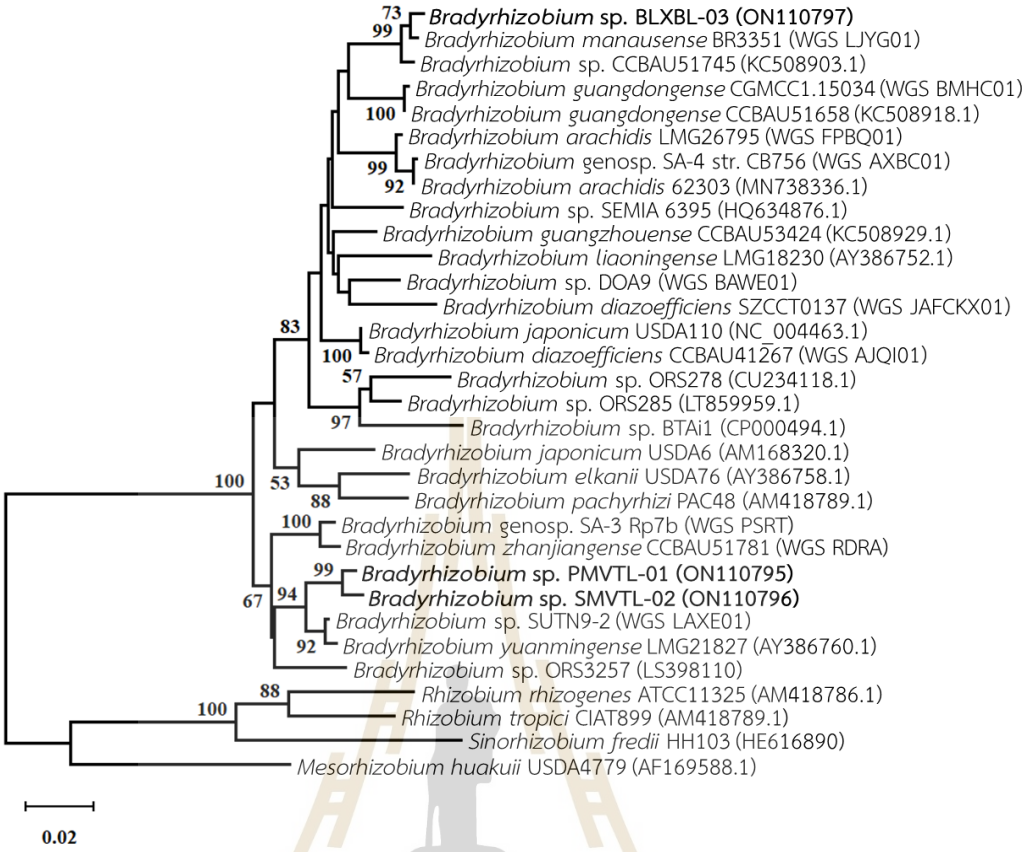

Fig 4.14 *atpD* gene phylogenetic tree, the phylogenetic tree procreated based on housekeeping genes were conducted in MEGA11, the bootstrap is calculated inferred from 1000 replicates and shown at the respective nodes for values >50%, the scale bar represents 0.02% sequence divergence.

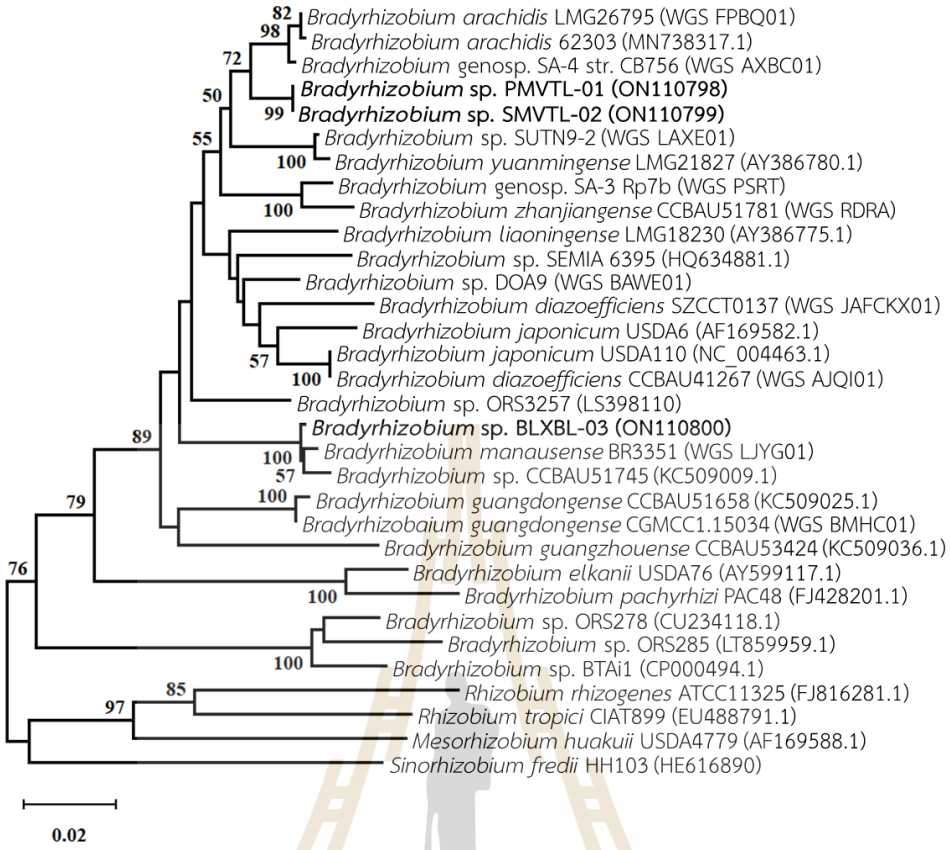

Fig 4.15 *glnII* gene phylogenetic tree, the phylogenetic tree procreated based on housekeeping genes were conducted in MEGA11, the bootstrap is calculated inferred from 1000 replicates and shown at the respective nodes for values >50%, the scale bar represents 0.02% sequence divergence.

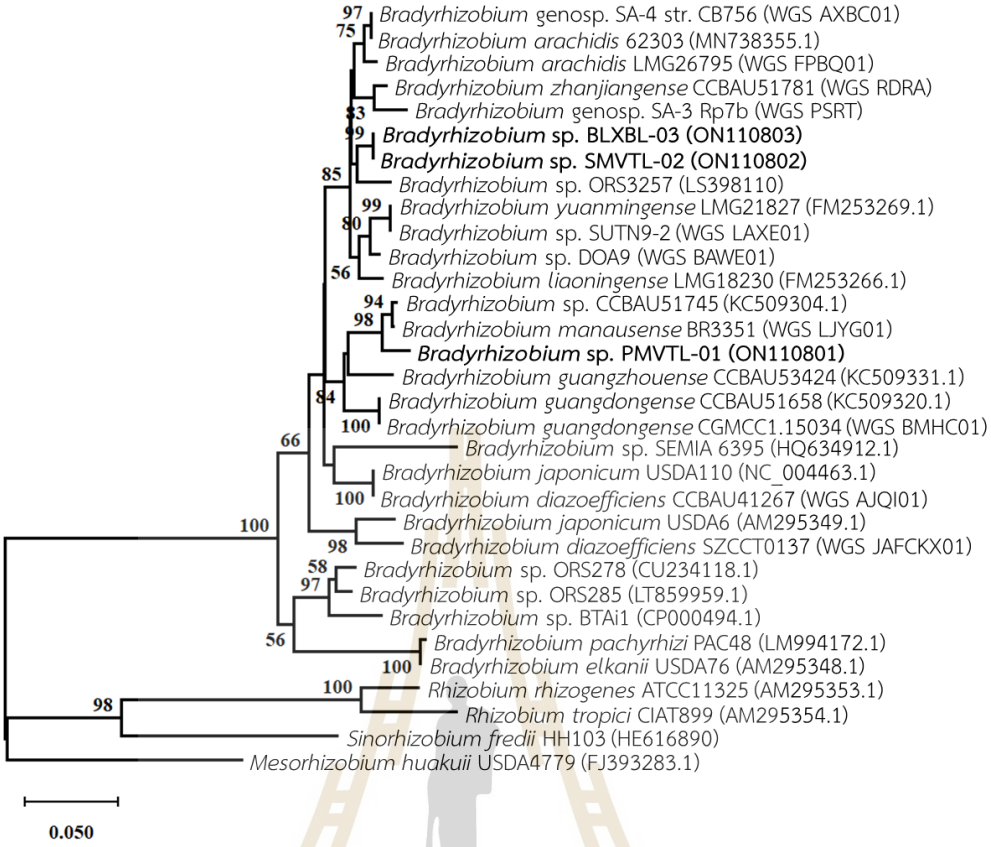

Fig 4.16 *rpoB* gene phylogenetic tree, the phylogenetic tree procreated based on housekeeping genes were conducted in MEGA11, the bootstrap is calculated inferred from 1000 replicates and shown at the respective nodes for values >50%, the scale bar represents 0.02% sequence divergence.

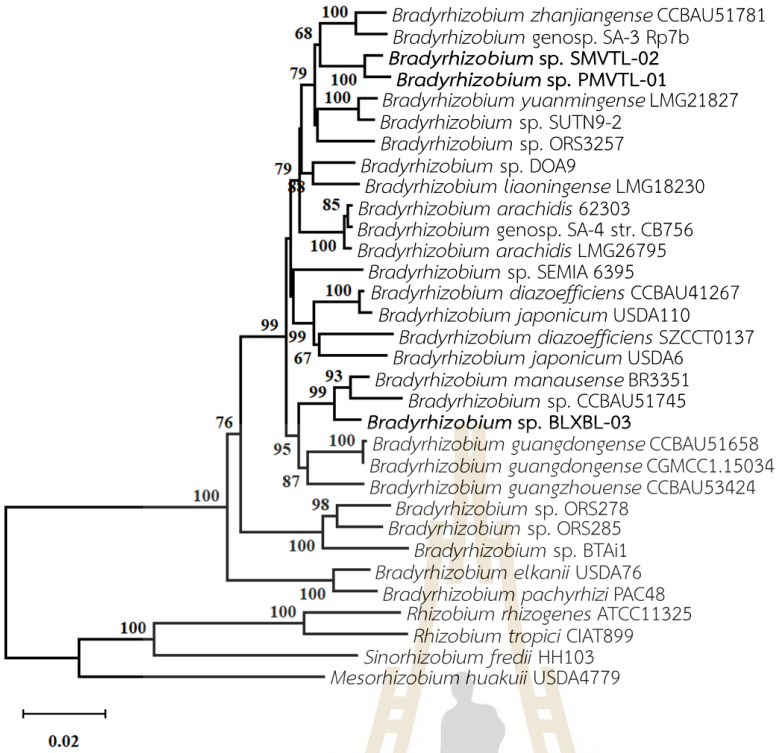

Fig 4.17 The sequences of combination five genes (16S rRNA, *recA*, *atpD*, *glnII* and *rpoB*) were combined in this phylogenetic tree, the phylogenetic tree was conducted in MEGA11, the bootstrap is calculated inferred from 1000 replicates and shown at the respective nodes for values >70%, the scale bar represents 0.02% sequence divergence.

4.7 Determination of competitiveness

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

To test the competitiveness ability of wild type strain PMVTL-01, SMVTL-02, and BLXBL-03 compared to *Bradyrhizobium* sp. strain SUTN9-2::GFP (Fig.4.18 A-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. 4.18 A 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 (64.24% SUTN9-2 and 35.76% SMVTL-02) (Fig. 4.18 B and E). In the case of BLXBL-03 showed lower nodules 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. 4.18 C and F). These findings indicated that the competitive ability of strain SMVTL-02 had a higher ability of nodules occupancy than 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. 4.7B), 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).

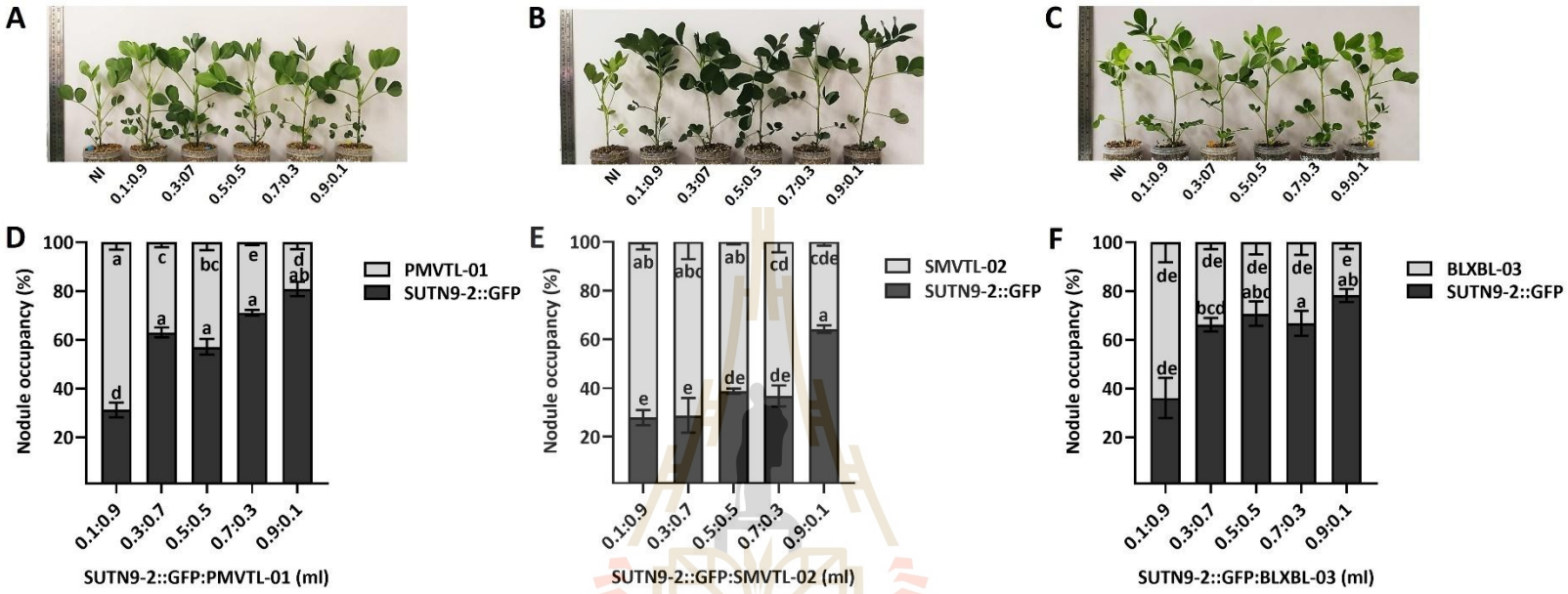

Fig 4.18 The competition assay among between *Bradyrhizobium* sp. strain PMVTL-01, SMVTL-02 and BLXBL-03 and SUTN9-2::GFP strain. (A-C) plant phenotype; (A and D) co-inoculated between PMVTL-01 and SUTN9-2::GFP, (B and E) co-inoculated between SMVTL-02 and SUTN9-2::GFP, and (C and F) co-inoculated between BLXBL-03 and SUTN9-2::GFP. Values with different letters in each treatment differed significantly at $P \geq 0.05$ (n = 3).

4.7.2 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. 4.19 A and B). The DsRed-expressing tagged strain showed higher nodules occupancy than indigenous strains (81.98% SMVTL-02::DsRed and 18.02% indigenous (Fig. 4.19 C). 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. 4.19 C-G).

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 experiments, while 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.

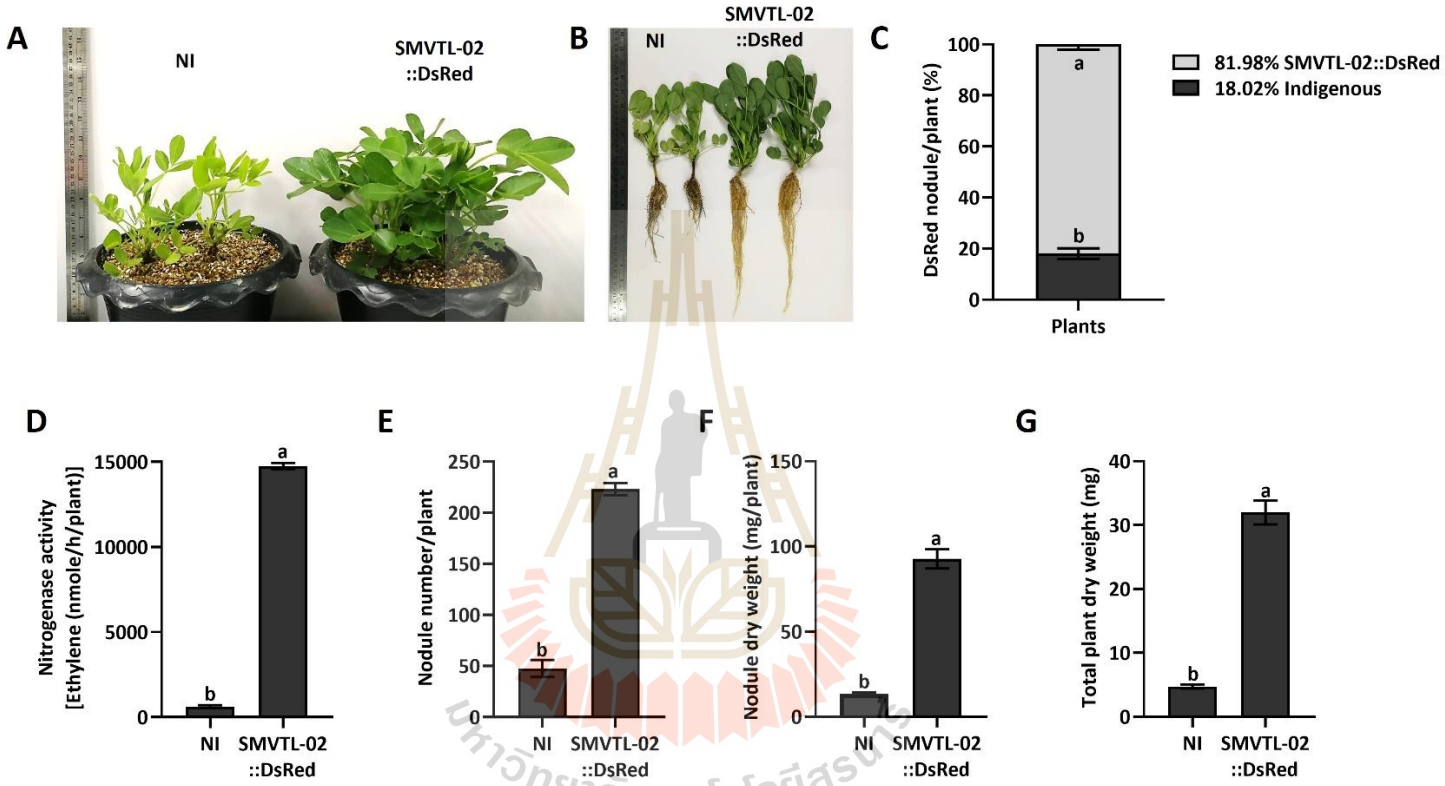

Fig 4.19 The competition assay among between *Bradyrhizobium* sp. strain SMVTL-02::DsRed and indigenous strain. (A-B) plant phenotype, (C) percent of nodules occupancy by SMVTL-02::DsRed and indigenous strains, (D) nitrogenase activity, (F) Nodule dry weight and (G) Total plant dry weight. The results were measured in peanut under lightroom conditions at 30 DAI, non-inoculated (NI). Values with different letters in each treatment differed significantly at $P \geq 0.05$ (n = 3).

CHAPTER V
CONCLUSIONS

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 including 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

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APPENDIX

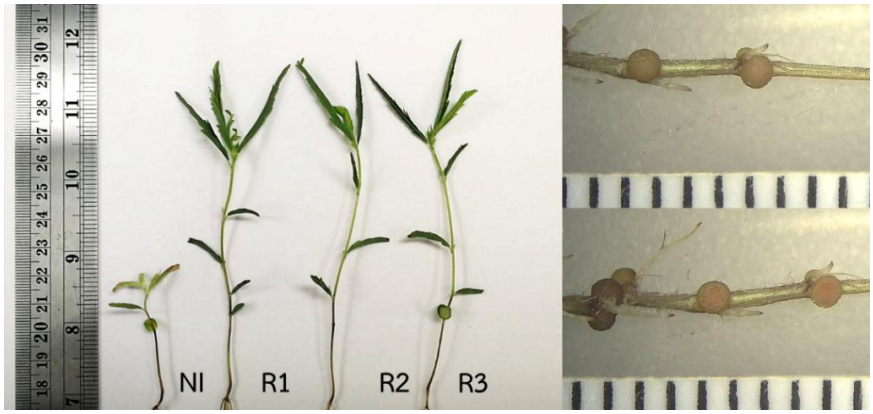

Fig A1. *Bradyrhizobium* sp. PMVTL-01 induced nodules on *A. Americana.*

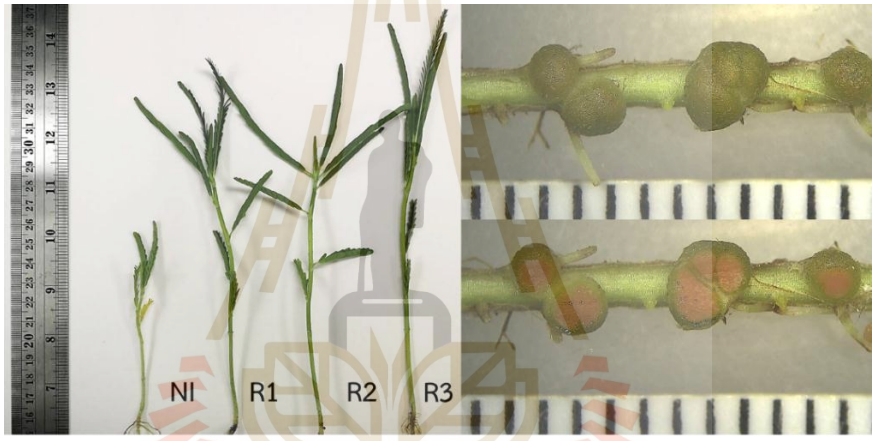

Fig A2. *Bradyrhizobium* sp. PMVTL-01 induced nodules on *A. afraspera*.

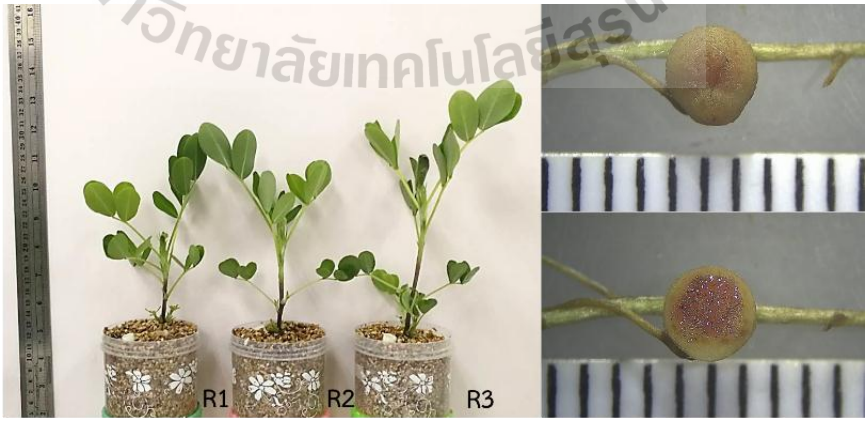

Fig A3. *Bradyrhizobium* sp. PMVTL-01 induced nodules on *A. hypogaea* (Thainan 9).

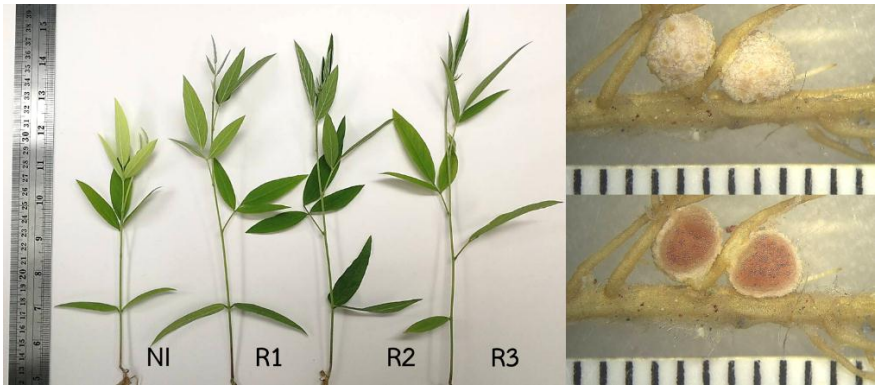

Fig A4. *Bradyrhizobium* sp. PMVTL-01 induced nodules on C. *cajan* (Linn.) Millsp.

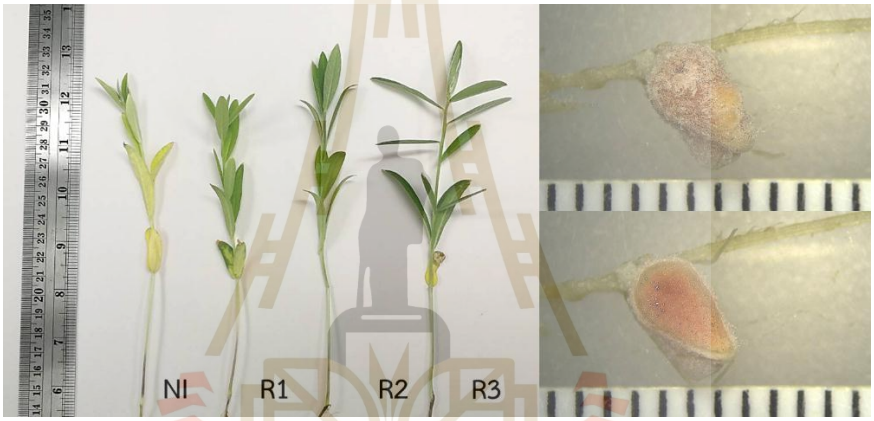

Fig A5. *Bradyrhizobium* sp. PMVTL-01 induced nodules on *C. juncea*.

Fig A6. *Bradyrhizobium* sp. PMVTL-01 induced nodules on *I. tinctoria*

Fig A7. *Bradyrhizobium* sp. PMVTL-01 induced nodules on *M. atropurpureum*.

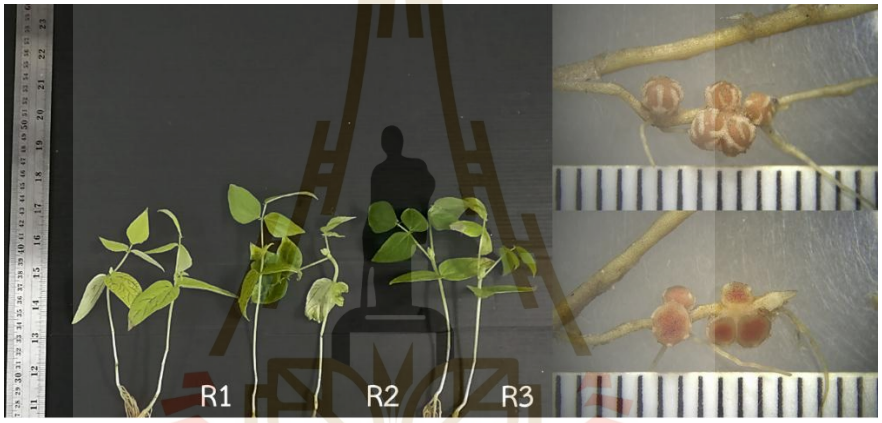

Fig A8. *Bradyrhizobium* sp. PMVTL-01 induced nodules on *V. radiata* (SUT1).

Fig A9. *Bradyrhizobium* sp. PMVTL-01 induced nodules on *V. mungo* (U thong 2).

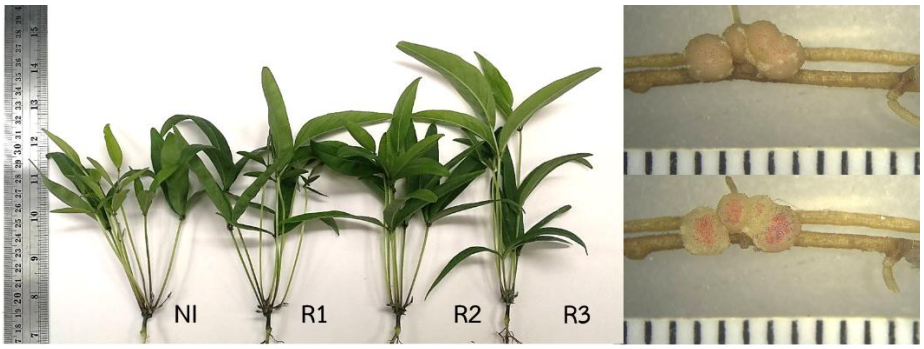

Fig A10. *Bradyrhizobium* sp. PMVTL-01 induced nodules on *V. subterranean*.

Fig A11. *Bradyrhizobium* sp. PMVTL-01 induced nodules on *V. unguiculata ssp. Sesquipedalis*

Fig A12. *Bradyrhizobium* sp. SMVTL-02 induced nodules on *A. Americana*.

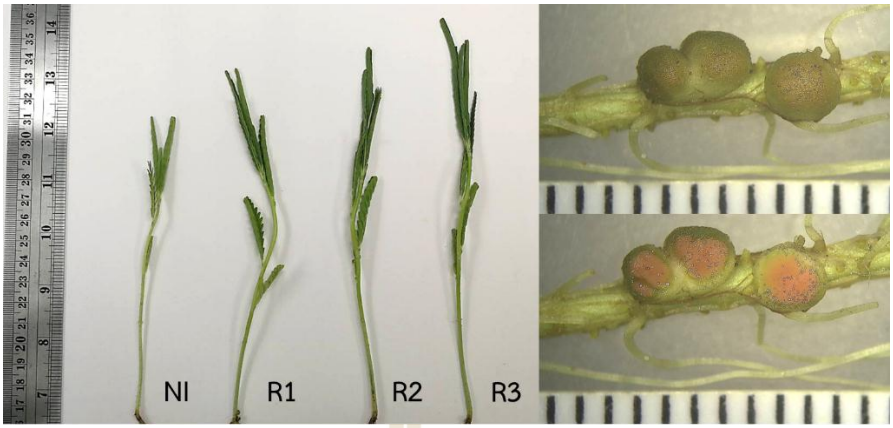

Fig A13. *Bradyrhizobium* sp. SMVTL-02 induced nodules on *A. afraspera*.

Fig A14. *Bradyrhizobium* sp. SMVTL-02 induced nodules on *A. hypogaea* (Thainan 9).

Fig A15. *Bradyrhizobium* sp. PMVTL-01 induced nodules on *C. juncea*.

Fig A16. *Bradyrhizobium* sp. SMVTL-02 induced nodules on *I. tinctoria*.

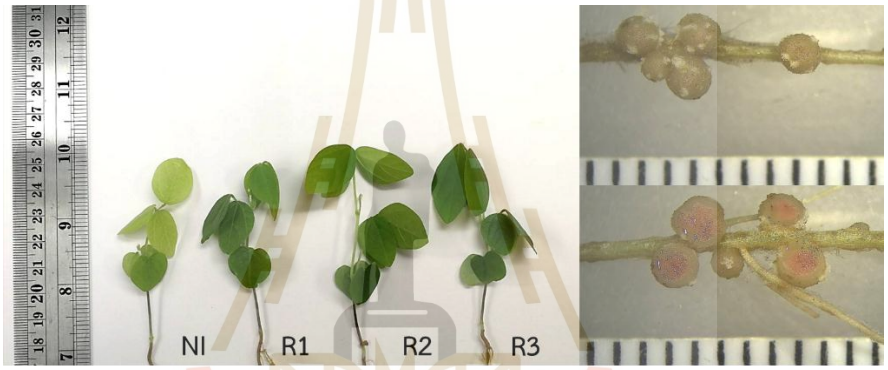

Fig A17. *Bradyrhizobium* sp. SMVTL-02 induced nodules on *M. atropurpureum*.

Fig A18. *Bradyrhizobium* sp. SMVTL-02 induced nodules on *V. radiata* (SUT1).

Fig A19. *Bradyrhizobium* sp. SMVTL-02 induced nodules on *V. mungo* (U thong 2).

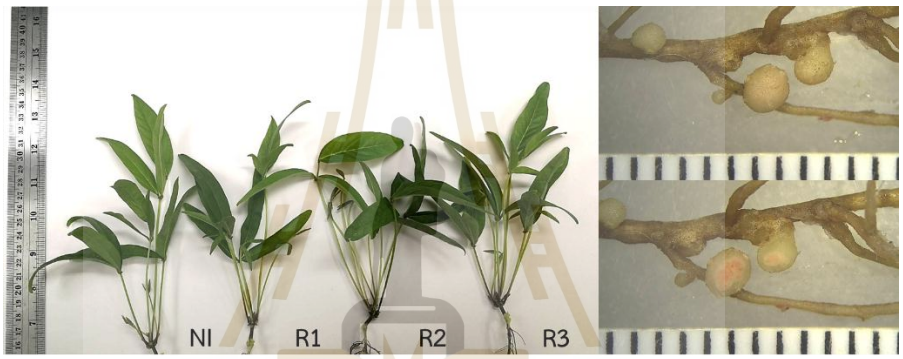

Fig A20. *Bradyrhizobium* sp. SMVTL-02 induced nodules on *V. subterranean*.

Fig A21. *Bradyrhizobium* sp. SMVTL-02 induced nodules on *V. unguiculata ssp. Sesquipedalis*

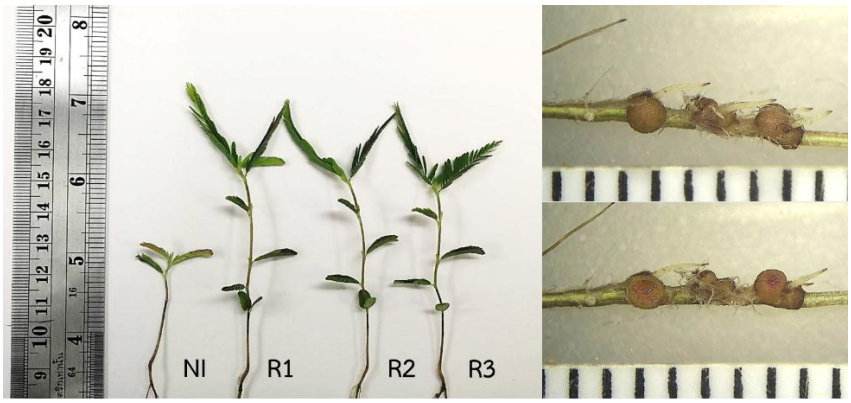

Fig A22. *Bradyrhizobium* sp. BLXBL-03 induced nodules on *A. Americana*.

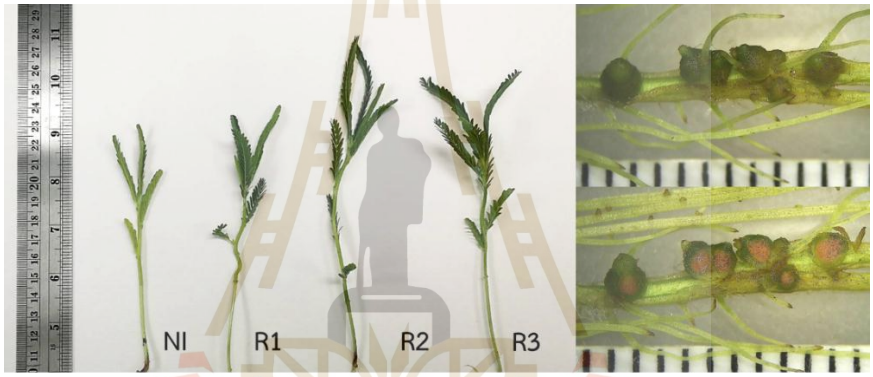

Fig A23. *Bradyrhizobium* sp. BLXBL-03 induced nodules on *A. afraspera*.

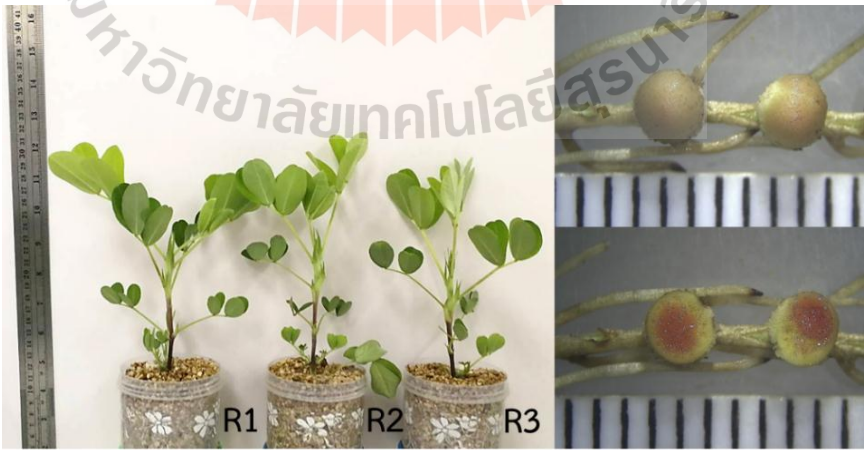

Fig A24. *Bradyrhizobium* sp. BLXBL-03 induced nodules on *A. hypogaea* (Thainan 9).

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Fig A25. *Bradyrhizobium* sp. BLXBL-03 induced nodules on C. *cajan* (Linn.) Millsp.

Fig A26. *Bradyrhizobium* sp. BLXBL-03 induced nodules on *I. tinctoria*.

Fig A27. *Bradyrhizobium* sp. BLXBL-03 induced nodules on *M. atropurpureum*.

Fig A28. *Bradyrhizobium* sp. BLXBL-03 induced nodules on *V. mungo* (U thong 2).

Fig A29. *Bradyrhizobium* sp. BLXBL-03 induced nodules on *V. subterranean*.

Fig A30. *Bradyrhizobium* sp. BLXBL-03 induced nodules on *V. unguiculata ssp. Sesquipedalis*.

BIOGRAPHY

Miss Tarnee Phimphong was born on December 1st, 1997 in Viengthong village, Hinherb district, Vientiane province and currently resides at Nontae village, Xaythany district, Vientiane capital, Lao PDR. In 2014, she graduated high school from Dongbang high school, Vientiane capital. She graduated with the Higher Diploma in TVET of English Language at Khamsavath College in 2007. In, 2018, she graduated with the Bachelor Degree of Plant Protection, Faculty of Agriculture, National University of Laos. Then, she studied Master Degree at School of Biotechnology, Suranaree University of Technology, Nakhon Ratchasima, Thailand in 2019-2022.