DEVELOPMENT RAPID TECHNIQUE FOR EVALUATION AND MONITORING RHIZOBIA

Chayanan Singtothong

A Thesis Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Biotechnology
Suranaree University of Technology
Academic Year 2023

การพัฒนาเทคนิคที่รวดเร็วในการประเมินและติดตามไรโซเบียม

นายชยานันท์ สิงห์โตทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2566

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of a Master's Degree.

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ชยานันท์ สิงห์โตทอง: การพัฒนาเทคนิคที่รวดเร็วในการประเมินและติดตามไรโซเบียม (DEVELOPMENT RAPID TECHNIQUE FOR EVALUATION AND MONITORING RHIZOBIA) อาจารย์ที่ปรึกษา: รองศาสตราจารย์ ดร. พรรณลดา ติตตะบุตร, 62 หน้า

คำสำคัญ: ไรโซเบียม/พืชตระกูลถั่ว/การตรึงไนโตรเจน/หัวเชื้อปุ๋ยชีวภาพแบบผสม/การควบคุมคุณภาพ/แอนติบอดี้ลูกผสม/เทคโนโลยีการแสดงแอนติบอดีบนผิวเฟจ

ไรโซเบียมเป็นแบคทีเรียชนิดหนึ่งที่มีความสามารถในการตรึงไนโตรเจน และมีความสำคัญมากในการใช้เป็นปุ๋ยชีวภาพสำหรับการผลิตพืชตระกูลถั่ว ทั้งนี้การใช้หัวเชื้อไรโซเบียมในรูปแบบหัวเชื้อผสมเป็นอีกวิธีหนึ่งที่ช่วยเพิ่มประสิทธิภาพของปุ๋ยชีวภาพเมื่อนำไปใช้ในหลากหลายพื้นที่ อย่างไรก็ตามการผลิตหัวเชื้อผสมนี้จำเป็นต้องมีเครื่องมือหรือวิธีการที่มีประสิทธิภาพในการตรวจสอบและติดตามเชื้อแต่ละชนิดที่นำมาผลิตได้อย่างรวดเร็วและแม่นยำเพื่อการควบคุมคุณภาพของผลิตภัณฑ์ สำหรับวิทยานิพนธ์นี้มุ่งเน้นการประยุกต์ใช้แอนติบอดีปรับแต่งพันธุกรรมชนิดเส้นเดี่ยวเอสซีเอฟวี (scFv) ที่คัดเลือกจากคลังแอนติบอดีมนุษย์ เป็นเครื่องมือในการตรวจสอบคุณภาพของปุ๋ยชีวภาพไรโซเบียมชนิดผสมที่ใช้สำหรับการปลูกถั่วลิสง โดยใช้หัวเชื้อผสมระหว่าง แบรดีไรโซเบียม สายพันธุ์ SUTN9-2 และ DASA03028 เป็นต้นแบบในการทดสอบ ทั้งนี้แบรดีไรโซเบียม สายพันธุ์ SUTN9-2 สามารถใช้แอนติบอดี yiN92-e10 ที่ได้จากงานวิจัยที่ผ่านมาสำหรับใช้ในการทดสอบ ในขณะที่วิทยานิพนธ์นี้ได้ทำการคัดเลือกแอนติบอดี yi028-F11 จากคลังแอนติบอดีมนุษย์ ในการจับจำเพาะกับแบรดีไรโซเบียม สายพันธุ์ DASA03028 โดยแอนติบอดีทั้งสองนี้ไม่มีปฏิกิริยาการจับข้ามต่อกัน และจากผลการทดสอบพบว่าแอนติบอดี yi028-F11 ปริมาณ 20 ไมโครกรัมต่อมิลลิลิตรสามารถใช้ในการตรวจจับแบรดีไรโซเบียม สายพันธุ์ DASA03028 ที่จำนวนเซลล์ตั้งแต่ 10^4 เซลล์ขึ้นไปได้ โดยพบว่าอายุโคโลนีของแบรดีไรโซเบียม สายพันธุ์ DASA03028 มีผลต่อความสามารถในการจับแบบจำเพาะของแอนติบอดี yi028-F11 ซึ่งเมื่อทดสอบโดยเทคนิคอีไลซา (Enzyme Linked Immunosorbent Assay; ELISA) พบว่าความสามารถในการจับจำเพาะของแอนติบอดีลดลงเมื่อโคโลนีมีอายุมากกว่า หรือน้อยกว่า 7 วัน อย่างไรก็ตามไม่พบว่าอายุโคโลนีของสายพันธุ์ SUTN9-2 มีผลต่อความสามารถในการจับจำเพาะของแอนติบอดี yiN92-e10 นอกจากนี้ยังประสบความสำเร็จในการนำแอนติบอดีชนิดนี้มาใช้ในเทคนิคฟลูออเรสเซนส์แอนติบอดี (Fluorescent antibody) สำหรับการตรวจสอบจำนวนเซลล์ที่ยังมีชีวิตของเชื้อแบรดีไรโซเบียมแต่ละชนิดทั้งในหัวเชื้อเดี่ยวและหัวเชื้อผสมหลังจากการเก็บรักษาทั้งในรูปแบบผลิตภัณฑ์หัวเชื้อเหลว และหัวเชื้อที่ใช้พีทเป็นวัสดุพาหะ อย่างไรก็ตามเมื่อประยุกต์ใช้เทคนิคดังกล่าวในการติดตามเชื้อแบรดีไรโซเบียมในรูปแบบแบคทีรอยด์ภายในปมถั่ว พบว่าไม่ประสบความสำเร็จกับถั่วลิสงทุกสายพันธุ์ที่นำมาทดสอบ แต่ประสบความสำเร็จในการทดสอบกับปมจากถั่วเขียว (*Vigna radiata*) ทุกสายพันธุ์ที่นำมาทดสอบ ผลการทดลองนี้แสดงให้เห็นถึงข้อจำกัดของการใช้แอนติบอดีปรับแต่งพันธุกรรมชนิดเส้นเดี่ยวเอสซีเอฟวี (scFv) ในการติดตามเชื้อแบรดีไรโซเบียมในพืชตระกูลถั่วบางชนิด อย่างไรก็ตามวิทยานิพนธ์นี้เป็นการริเริ่มใช้

แอนติบอดีปรับแต่งพันธุกรรมชนิดเส้นเดี่ยวเอสซีเอฟวี (scFv) เป็นครั้งแรกสำหรับการตรวจสอบจำนวนของแบรดีไรโซเบียมที่ยังมีชีวิตโดยสามารถจำแนกสายพันธุ์ของเชื้อได้อย่างแม่นยำในหัวเชื้อรูปแบบผสม

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2566

ลายมือนักศึกษา.....
ลายมืออาจารย์ที่ปรึกษา.....
ลายมืออาจารย์ที่ปรึกษาร่วม.....
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ลายมืออาจารย์ที่ปรึกษาร่วม.....

CHAYANAN SINGTOTHONG: DEVELOPMENT RAPID TECHNIQUE FOR EVALUATION AND MONITORING RHIZOBIA. THESIS ADVISOR: ASSOC. PROF. PANLADA TITTABUTR, Ph.D., 62 PP.

Keyword: *Rhizobium*/Legume plants /Nitrogen fixation/Mixed culture biofertilizer/Quality control/Recombinant scFv/Phage display

Rhizobium, a nitrogen-fixing bacterium, plays an important role in legumes plant production as biofertilizer. To enhance nodulation and nitrogen fixation success in diverse field locations, mixed-culture of effective rhizobia has emerged as a promising strategy. Consequently, developing a robust technique for precise detection and continuous monitoring of each bradyrhizobial strain is necessary for quality control. This study focuses on the application of recombinant human scFv antibodies as a quality control technique for peanut bradyrhizobial mixed-culture inoculant. The mixed-culture inoculant of *Bradyrhizobium* sp. SUTN9-2 and *Bradyrhizobium* sp. DASA03028 was used as a model in this study. A specific scFv antibody yiN92-e10 for strain SUTN9-2 has been previously characterized, while a specific scFv antibody yi028-F11 targeting strain DASA03028 was selected through biopanning in this study from a naïve human scFv antibody phage display library. There was no cross-binding reactivity between these two scFv antibodies and among other tested bradyrhizobia. The scFv antibody yi028-F11 at concentration of 20 µg/ml was able to detect 10^4 cells of strain DASA03028. The colony age of strain DASA03028 influenced the antibody binding affinity, the colony age younger or older than 7 days after incubation showed reduction in the signal via ELISA detection. However, no influence of colony age on binding activity for antibody yiN92-e10 with strain SUTN9-2. These recombinant scFv antibodies were successfully employed with the fluorescent antibody (FA) technique to assess viable cell counts of individual bradyrhizobial strains in both liquid and peat-based single and mixed-culture inoculants. Unfortunately, bacteroid detection in peanut nodules using these antibodies was unsuccessful in all tested peanut cultivars, but it was successful for the detection in mung bean (*Vigna radiata*) nodules regardless of mung bean cultivars. These results indicate the limitation of using scFv antibody with some legumes. Nevertheless, this study introduced for the first time of using

scFv antibodies for detection of individual viable bradyrhizobial strain in mixed-culture situation.

School of Biotechnology
Academic Year 2023

Student's Signature.....
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ACKNOWLEDGEMENTS

This work was supported by the One research One Graduate (OROG) of The Thailand Research Fund, Agricultural Research Development Agency (Public Organization) (ARDA) (Acardemic year 2021) (HRD6405070 / M6200121-2/2564) and the School of Biotechnology, Institute of Agriculture Technology, Suranaree University of Technology.

I would like to express my deepest sense of gratitude to my advisor Assoc. Prof. Dr. Panlada Tittabutr and my co-advisors, Prof. Dr. Montarop Yamabhai, who has been suggestively supportive my learning goal and worked inspirable to promote me with laboratory experience and academic writing in paper and thesis book.

Furthermore, I would like to thank Dr. Monchai Manassila and Department of Agriculture (DOA) for purpose of these thesis and source of peanut bradyrhizobia.

I express my deep sense of gratitude to Sarun Petchmark and Polina Istomina for their help the affinity selection and protein production.

Then, I would like to thank Dr. Kamonluck Teamtisong, Dr. Pongdet Piromyou, Dr. Pongpan Songwattana, Dr. Jenjira Wongdee, Dr. Teerana greetatorn, Asst. Prof. Dr. Pakpoom Boonchuen and Dr. Kuntalee Rangnoi who provide the equipments, helping lab work, and suggested lab work.

Finally, my warm thank are due to my family and my closely gratitude to the member of NPN lab, MY lab and PB lab who have been guidance of my education, and suggested me even in the hard time during my study.

Chayanan Singtothong

CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

A	=	Absorbent
ACC	=	1-aminocyclopropane-1-carboxylic acid
Amp	=	Ampicillin
bp	=	Base pairs
BSA	=	Bovine serum albumin
CFU	=	Colony forming units
CDR	=	Complementary determining region
DNA	=	Deoxyribonucleic acid
DOA	=	Department of Agriculture
E. coli	=	*Escherichia coli*
ELISA	=	Enzyme-linked immunosorbent assay
et al.	=	et alia (and other)
FA	=	Fluorescent Antibody
GFP	=	green fluorescent protein
HRP	=	Horse radish peroxidase
IgG	=	Immunoglobulin class
IAA	=	indole acetic acid
mM	=	millimolar
MPN	=	Most Probable Number
N	=	nitrogen
N_2	=	gas nitrogen
OD	=	Optical density
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffer saline
RNS	=	Root-nodule symbiosis
RT	=	Room temperature
scFv	=	Single chain Fv antibody derivative
SDS	=	Sodium dodecyl sulphate

LIST OF ABBREVIATIONS (Continued)

T3SS	=	type III secretion system
UV	=	Ultraviolet
µg	=	Microgram
kDa	=	(kilo) Daltons
µl	=	Microliter
°C	=	degree Celsius
g	=	grams
h	=	hours
Kg	=	kilograms
L	=	liter
m	=	meter
M	=	molar
mg	=	milligram
min	=	minute
ml	=	milliliter
rpm	=	rounds per minute
sec	=	seconds
v/v	=	volume per unit volume
w/v	=	weight per unit volume
YM	=	yeast extract mannitol

CHAPTER I

INTRODUCTION

1.1 Background

Leguminous plant as peanut or groundnut (*Arachis hypogaea*) is rich in nutrients, especially protein and also used for cooking oil production (Sistani and Morrill, 1989). To get high efficiency crop production and reduce cost of chemical fertilizer, the application of nitrogen biofertilizer *Bradyrhizobium* is necessary. *Bradyrhizobium* sp. is one of soil bacteria that can convert atmospheric nitrogen into ammonia and provide nitrogen to peanut for growth requirement (Bogino et al., 2006). In some cases, a selected single strain of rhizobial inoculant that can significantly improve legume crop growth under controlled condition, fail to compete against native strains or fail to encounter with the environmental condition when applied in the field soil and resulting in reducing the N_2 fixing amount of legume plant. This situation has known as the rhizobial competition problems. In order to solve these problems, the biofertilizer with mixed culture of rhizobia is one of the strategies to increase the chance of legume nodulation by competing with soil indigenous strains or encountering the soil stress condition. However, to produce the mixed culture inoculant, it is important to ensure that all strains used for production are in high amount of living cells, and having less or no contamination (Lupwayi et al., 2000). Therefore, it is important to develop the robust quality control technique to determine the number of living cell for each bradyrhizobial strain used in the mixed culture inoculant during the production and after storage to ensure the successful nodulation and symbiosis with host legume in the field application.

In Thailand, Department of Agriculture (DOA) is responsible for rhizobial inoculant production and development. For peanut, several strains of *Bradyrhizobium* sp. which showed high performance on peanut symbiosis were recommended by DOA and generally used for mixed culture inoculant production for peanut. The strains used for mixed culture inoculant, such as DASA03156, DASA03118, DASA03084, DASA03094, and DASA03028. However, production of mixed culture always face with the contamination problem and it is difficult to determine the living cell amount of each strain during the inoculant production or after storage. DOA usually use the standard technique of plant infection

method to estimate the number of total rhizobia in the inoculant based on the Most Probable Number (MPN) of nodule formed by rhizobia at different cell dilution. This technique takes at least 3-4 weeks (Senthilkumar et al., 2021) to determine the result and it could not interpret the cell number of each bradyrhizobial strain mixed in the inoculant. Therefore, the efficient technique should be developed to control the quality of mixed culture inoculant.

The serological technique especially Enzyme-linked immunosorbent assay (ELISA) and Fluorescent Antibody (FA) technique have been used as the basic tools for evaluation and monitoring the rhizobia. ELISA is one of the popular enzyme immunoassays used in detecting antigens. Advantages of this technique are the specificity, rapid and no microscopic equipment needed. However, FA technique is one of the most sensitive serological methods available and has been used for robust detection of rhizobia (Vu et al., 2017). Advantage of this technique is the ability to be used as a high throughput method for identify bradyrhizobia in large numbers. However, the traditional way for producing antibody must use the application of immune system in rabbit to produce polyclonal antibody (Wilson et al., 1988) for each rhizobium. The main problem of using polyclonal antibody to detect and monitor specific rhizobium is very often to have cross--reactivity with other rhizobial strains within the same species (Vu et al., 2017). Therefore, in this research the application of phage display technology was used for the generation of monoclonal antibody for specific detection of bradyrhizobium.

In this research, the peanut *Bradyrhizobium* sp. strains provided from DOA are selected to develop the specific antibody using phage display technology and further developed as a mixed culture with *Bradyrhizobium* sp. strain SUTN9-2. *Bradyrhizobium* sp. SUTN9-2 has been used as selected strain for peanut inoculant, which is produced under Laboratory of Inoculant production research group, Suranaree University of Technology (SUT) (Noisangiam et al., 2012). This strain produced plant hormone, indole acetic acid (IAA) (Greetatorn et al., 2019), and contain 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity that can decrease plant stress under water deficit condition (Sarapat et al., 2020). The specific antibody for the strain SUTN9-2 using phage display technology has been produced and characterized (Khaing et al, 2021). The strain SUTN9-2 in combination with the recommended strain from DOA as mixed culture inoculant was used as a model in this study. This research aimed at development of robust technique

for observation the living cell number of each bradyrhizobium strain during production and storage of mixed culture inoculant as well as for monitoring the strain in nodules after applied to legume. Therefore, the specific and precise technique is required in order to improve the quality control of peanut inoculant production and ensure the nodulation efficiency with this legume.

1.2 Research objectives

1.2.1 To apply phage display antibody technology for the development of robust technique in detection and monitoring of rhizobia.

1.2.2 To evaluate the viable cell number of the specific *Bradyrhizobium* strains contain in mixed culture inoculant and monitor their nodulation ability with legumes.

Specific objectives of this study were:

1. To affinity select phage displayed scFv antibodies which are specific to the selected peanut *Bradyrhizobium*
2. To produce recombinant scFv antibody in Escherichia coli and develop the technique for detection
3. To apply the scFv antibodies for evaluation the living cells of specific *Bradyrhizobium* contained in the mixed culture inoculant
4. To apply the scFv antibodies for monitoring the nodule occupancy of each specific *Bradyrhizobium* after mixed culture inoculation

1.3 Hypothesis

This study hypothesized that the selected phage displayed scFv antibody is specific to peanut bradyrhizobium and could be applied for evaluation the living cell number of the specific Bradyrhizobium in the mixed culture inoculant and used for monitoring the nodule occupancy of specific *Bradyrhizobium* in the legume nodule

1.4 Scope of the study

This study was focused on development the robust and specific technique for evaluation the living cell number of the mixed culture bradyrhizobia in liquid- and peat-based inoculant during production and after storage for quality control check purpose. This technique was developed based on the application of Enzyme-linked immunosorbent assay (ELISA) and fluorescent antibody (FA) techniques using the monoclonal antibody generated from phage display technology that specific for the selected peanut bradyrhizobia. The efficient peanut bradyrhizobia derived from DOA were used to screen the monoclonal antibody by performing the biopanning of phage display technology to affinity select phage displayed the single-chain variable fragments (scFv) antibodies specific to peanut *Bradyrhizobium*. The clone that shows highest affinity of phage display scFv and highly specificity was selected and later produced the scFv in *E. coli*. Then, one selected strain of peanut *Bradyrhizobium* provided by DOA was mixed with *Bradyrhizobium* sp. SUTN9-2 as a model of mixed culture inoculant for peanut in this study. The scFv-ELISA and -FA techniques were applied as a robust technique to determine the living cell number of the specific *Bradyrhizobium* in the mixed culture inoculant of both liquid- and peat-based inoculant forms and monitor the nodule occupancy of specific *Bradyrhizobium* in the legume nodules.

CHAPTER II

LITERATURE REVIEWS

2.1 Importance of Legume plants.

Legumes are a diverse group of plants that play a critical role in agriculture and the environment. Their unique ability to symbiosis with rhizobia and fix nitrogen from the atmosphere sets them apart from most other plants and offers a multitude of benefits (Graham and Vance, 2003). Legumes are grown on over 170 million hectares of land worldwide, making them one of the most important crop families (FAOSTAT, 2022). Legume plants such as soybeans hold a strong position among the top five globally consumed crops, following rice, wheat, maize, and potatoes. Their popularity stems from both direct consumption and their widespread use in various products (Daryanto et al., 2017).

Nitrogen (N) is always deficient in crop production. Application of nitrogen fertilizer results in the protein concentration in plant and biomass are increased (Kumar, 2014). Moreover, increasing of nitrogen availability to legume plant can also be increased by nitrogen fixing bacteria, normally called rhizobia, especially in genus of *Bradyrhizobium* (Wani et al., 1995).

2.2 Rhizobia

Rhizobia (the fast-growing *Rhizobium* spp. and the slow-growing *Bradyrhizobium* spp.). They are gram-negative bacteria, rod shape size 1.2-3.0 µm in length and 0.5-0.9µm in width. Rhizobia are predominantly aerobic chemoorganotrophs which optimal temperature for growing of most strains is 25-30 °c and a pH for 6.0-7.0. Normally, most rhizobia have white colony when culture on yeast extract mannitol (YM) medium. One of characteristics in most rhizobia is weakly absorb congo red (diphenyldiazo-bis-anaphthylaminesulfonate) dye, which is included in culture medium for rhizobia isolation (Mansour et al., 2023).

The free-living rhizobia in the soil can enter to the root hairs of legume plant (Somasegaran and Hoben, 2012). Root-nodule symbiosis (RNS) is started from the process of rhizobia invasion to root epidermis, and nodule organogenesis across the root cortical cells. The strategy of rhizobia invasion is to curling root hair and infection thread formation, while the primordia nodule is induced from a distance. However, there is alternative way of rhizobia invasion, crack-entry, while rhizobia enter to intercellular route at the lateral root base **(Fig. 2.1)**. Around 25% in approximately of legume plants are adapted to crack-entry mode, which is a characteristic application for some subtropical legumes, such as *Aeschynomene* sp., *Stylosanthes* sp., and *Arachis* sp. (Zhao et al., 2023). These rhizobia directly access to the cortical cells for nodule primordia development, and the infected cells divided repeatly from uninfected cells. Moreover, the type III secretion system (T3SS) which is known to play a role for delivery of virulence factors similar to pathogenic bacteria to symbiosis is one way that promote nodulation (Okazaki et al., 2013) that hijacking of leguminous nodulation signaling can be success by the rhizobial type III secretion system. However, recently some Type 3 effector proteins are also reported to have a negative effect on legume nodulation (Songwattana et al., 2017).

Rhizobia are group of diazotrophs belonging into the class of α-proteobacteria that including with the genera *Rhizobium*, *Shinella*, *Bradyrhizobium*, *Mesorhizobium*, *Azorhizobium*, *Sinorhizobium*, *Phyllobacterium*, *Methylobacterium*, *Devosia,* and *Ochrobactrum* (Allito et al., 2015). It is a good mentioning that *Bradyrhizobium* is a basal position of all rhizobia (Sharma et al., 2020). *Bradyrhizobium* sp. is a slow growing group of gram-negative soil bacteria root nodule symbiont (D. C. Jordan and Allen, 1974) that can fix atmospheric nitrogen into bioavailable nitrogen for plant as ammonia which is a non-limited source of nitrogen (Hartmann and Amarger, 1991) that widely used as biofertilizer inoculant for legume crop around the world. Normally, peanut is considered to be nodulated by the genus of *Bradyrhizobium* (Zhang et al., 2016). Moreover,

Bradyrhizobium can be nodulated in widely commercial legume plant, such as mungbean, soybean, cowpea, and others.

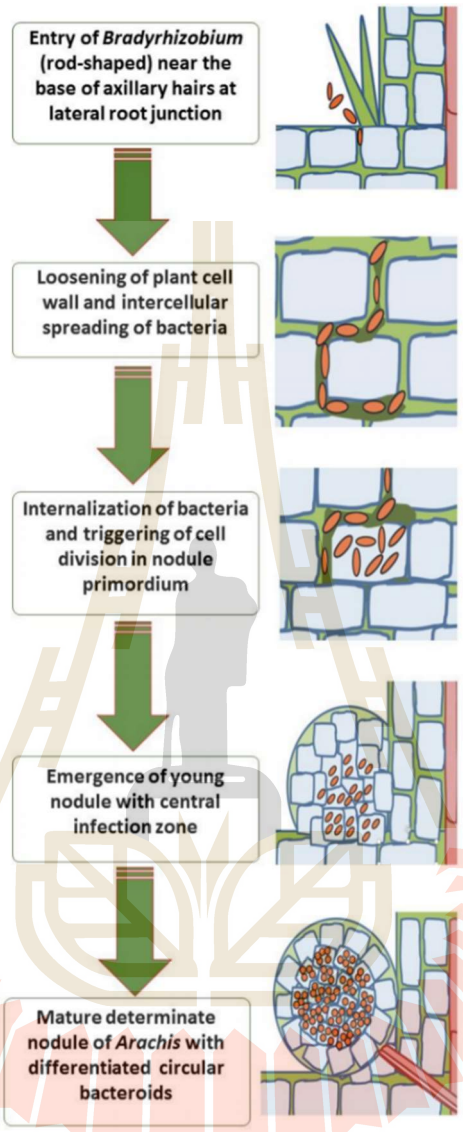

Figure 2.1 Successive stages of nodule development in groundnut and "crack-entry" invasion of *Bradyrhizobium* (Sharma et al., 2020).

2.3 Improvement of peanut crop with Bradyrhizobium inoculation

Nitrogen is one of the most essential nutrients for plant and a major factor that limiting plant crop productivity around the world. The maximization of biological nitrogen fixation (BNF) for different crop species are requirements in sustainable agriculture crop production (Sharma et al., 2020). BNF is necessary efficient production of leguminous crops as peanut. Although there are native bradyrhizobia present in the soil in some areas,

farmers still inoculate peanut with BNF in all field to ensure the successful colonization and nodule formation of roots. Inoculating of *Bradyrhizobium* in peanut can promote nitrogen fixation and nodulation (Hashem et al., 1997) and decrease the requirements for chemical nitrogen fertilizer (Bogino et al., 2006). The efficiency of inoculation with *Bradyrhizobium* on peanut was reported by Jordan et al., (2018) that liquid or granular inoculant can increase yield from 3,460 to 4,660 lb/acre in new peanut crop and 4,280 to 4,450 lb/acre in area with a previous history plantings peanut within the past 4 years. However, the efficiency of commercially formulated inoculant of *Bradyrhizobium* can be affected by environmental factors, indigenous bradyrhizobia, and soil conditions management (Jordan et al., 2009). Therefore, bacterial strain selection and inoculant production are necessary to overcome the adverse factors presented in the soil after inoculation.

2.4 Production of rhizobia legume inoculant

2.4.1 Strain selection for legume inoculant

Strain selection is important for quality purpose of inoculant production. Quality in selection strain of inoculant means that isolation of pure strain in which effectiveness and competitiveness for nodule formation should have been characterized (Lupwayi et al., 2000). The strain selection for legume inoculant could also be considered in other properties to promote legume production, such as ACC deaminase activity, indole acetic acid production, phosphate solubilization, and antagonistic activity (Yuttavanichakul et al., 2012). For peanut inoculant production, in this cases, *Bradyrhizobium* sp. strains DASA03118, DASA03028, DASA03094, DASA03028, and DASA03156 were obtained from the Thailand Department of Agriculture (DOA). These strains are recommended as efficient microsymbionts for peanut inoculation and were used in this study. Another strain used in this study is the *Bradyrhizobium* sp. strain SUTN9-2. This strain was isolated from nodule of *Aeschynomene americana* that grown in rice field areas in Thailand (Noisangiam et al., 2012). It has the capability of plant growth promotion characteristics, such as indole-3-acetic acid (IAA) production (Greetatorn et al., 2019) and support mung bean symbiosis under water deficit condition by increased 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase productions (Sarapat et al., 2020). This strain is also having capability to symbiosis well with peanut and generally used for peanut inoculant when produced by School of Biotechnology, SUT as requested from farmers. Moreover, the *Bradyrhizobium*

sp. strain SUTN9-2 was applied to produce specific antibody and used as a powerful tool to monitor nitrogen fixing biofertilizer in rice and legume (Khaing et al., 2021). Therefore, this strain was used in this study to mixed with one of the selected *Bradyrhizobium* provided from DOA to make a mixed culture inoculant as a model for investigation.

2.4.2 Inoculant formulations

The categories of rhizobium inoculant formulations can be summarized as Soil-based inoculant: (peat, coal, clays, and inorganic soil), Agar inoculant, Polymers inoculant, Lyophilized inoculant, and Liquid inoculants (Bashan et al., 2014). However, the way that DOA produces inoculant nowadays is in from of liquid inoculants (personal communication with Dr. Monchai Manassila). The main advantages of liquid inoculant are easier than solid inoculant to handle, and its shelf life could be more than 2 years (Bashan et al., 2014). The most limiting of liquid inoculant is the cool or cold temperature condition is required for long-term storage, resulting in increased the storage cost and limits their use in most developing countries (Stephens and Rask, 2000). However, inoculation with single strain rhizobium may not enough (Aryal et al., 2003). Inoculation with mixed culture rhizobia is one of strategies to make a chance of competitiveness and effectiveness to promote legume plant crop which showed positively influenced shoot, root dry weights and nodulation of plant in dually inoculated plants compared to singly inoculated plants. It is same as the way that DOA recommended to use mixed culture liquid inoculant.

DOA produced mixed culture of bradyrhizobia by mixing strains in inoculant to make more chance of symbiosis with peanut under field soil condition. However, there is no any specific and non-GMO tools used by DOA to evaluate the symbiosis efficiency or the competitiveness ability of each *Bradyrhizobium* strain to the native strain in the soil. Moreover, in case of inoculant production process, there is no specific tools to observe the cell number of each *Bradyrhizobium* strain in liquid inoculant to ensure the survival of all strains until inoculant was used. Based on these reasons, the specific technique for detection *Bradyrhizobium* especially in mixed culture inoculant is necessary.

2.5 Detection technique for Rhizobium

There are several different methods for detection of *Rhizobium*, such as homology of amplified DNA, biochemical -, protein -, antibiotic -, plasmid profiles, and serological properties (Nick and Lindström, 1994). *Rhizobium* strain tagging with reporter genes, such as green fluorescent protein (GFP) is also used for monitoring rhizobium when interaction

with plant but somehow unacceptable to be used in general because GMO reason, while serology or immunological technique that can be used for detection of rhizobia as a non GMO with a simple and rapid technique when compared to microbiological or DNA-based techniques (Vu et al., 2017). The applications of some techniques for detection of bradyrhizobia are reviewed below.

2.5.1 Green fluorescent protein (GFP)-marked strains of *Bradyrhizobium*

Green fluorescent protein (GFP) is a protein that found in the jellyfish (*Aequorea victoria*). It has capability to fluorescing when excited with UV light. Bacteria that tagged with the *gfp* gene is simple and easily to identify fluorescing colonies under UV light. The advantage of GFP marker is not require any substrate and no background activity is presented in indigenous soil microorganisms and plants. The visualization of *Bradyrhizobium* spp. in the rhizosphere and on root surfaces used tagged bradyrhizobial with GFP marker that constructed from strains of *Pseudomonas fluorescens* and *Enterobacter gergoviae* has been introduced for this purpose (Bhatia et al., 2002). Evaluation of nodule occupancy was done with tagged rhizobia as reported by Ramos et al., (2007), the reporter genes *gfp* and *gusA* was used to evaluate nodule occupancy of *Rhizobium tropici* in *Phaseolus vulgaris* nodules. It can therefore be used as a method for determining the nodule occupancy and have advantage as rapidly, inexpensive, and reliable. However, this technique can be used only in the laboratory because GMO in Thailand is not acceptable, and requires fluorescent microscopy equipment (Vu et al., 2017).

2.5.2 PCR-Amplified 16S rRNA genes

Comparison of 16S rRNA nucleotide sequences and phylogenetic approach based on DNA-rRNA hybridization can be applied to classify of isolates at the species and higher levels. Comparison of 16S rRNA genes sequences has been used to classify differentiation between *Rhizobium* species of DNA sequencing (Laguerre et al., 1994). However, these methods are time-consuming and thus are not appropriate for routine identification (Vu et al., 2017).

2.5.3 The immunological techniques

Affinity of antigen and antibody is basic for immunological techniques, as when bacteria are infecting to a mammal (including rhizobia), the animal has an immune system which produce antibodies that has affinity to antigens and bind on the surface of the

bacteria. The study of immune serum reaction with the antigens outside the animal is known as serology. Detection and identification of the target rhizobia can be applied base on binding of the antibodies, if the surface of rhizobium antigen is relatively unique and unshared by other microorganisms (Vu et al., 2017).

Antibody can be divided into polyclonal antibody and monoclonal antibody. Generally, polyclonal antibody is widely used to monitor and detect specific rhizobium. Polyclonal antibody was constructed by application of mammal immune system, such as rabbit or mouse (Wilson et al., 1988), which have limitation as cross-reactivity to other rhizobia within the same or close species. It means that using polyclonal antibody for immunological techniques is not specific. Therefore, monoclonal antibody is necessary. Monoclonal antibody is very specific because it made form cloning of a unique white blood cell, which have affinity to binding only the same epitope. Hybridoma technology can be used to generate monoclonal antibody which discovered in 1975 and Noble prize award in 1984 for physiology and medicine (Pandey, 2010). Phage display technique which first described by George P Smith in 1985, and Noble prize award in 2018. This technique, the bacteriophage is genetically modified to display peptides and proteins (Barderas and Benito-Peña, 2019). Moreover, phage can display a smallest unit of immunoglobulin molecule with function in antigen-binding activities as single chain fragment variable (scFv). The scFv format consists of variable regions of heavy (VH) and light (VL) chains, that joined together with a flexible peptide linker. The *E. coli* can be used as a host to overexpress this protein which have function of antigen-binding activities (Griffiths and Duncan, 1998). The schematic of using phage display technology to produce monoclonal antibody was described in **Fig. 2.2** and more detail are described in the section **2.6.2.**

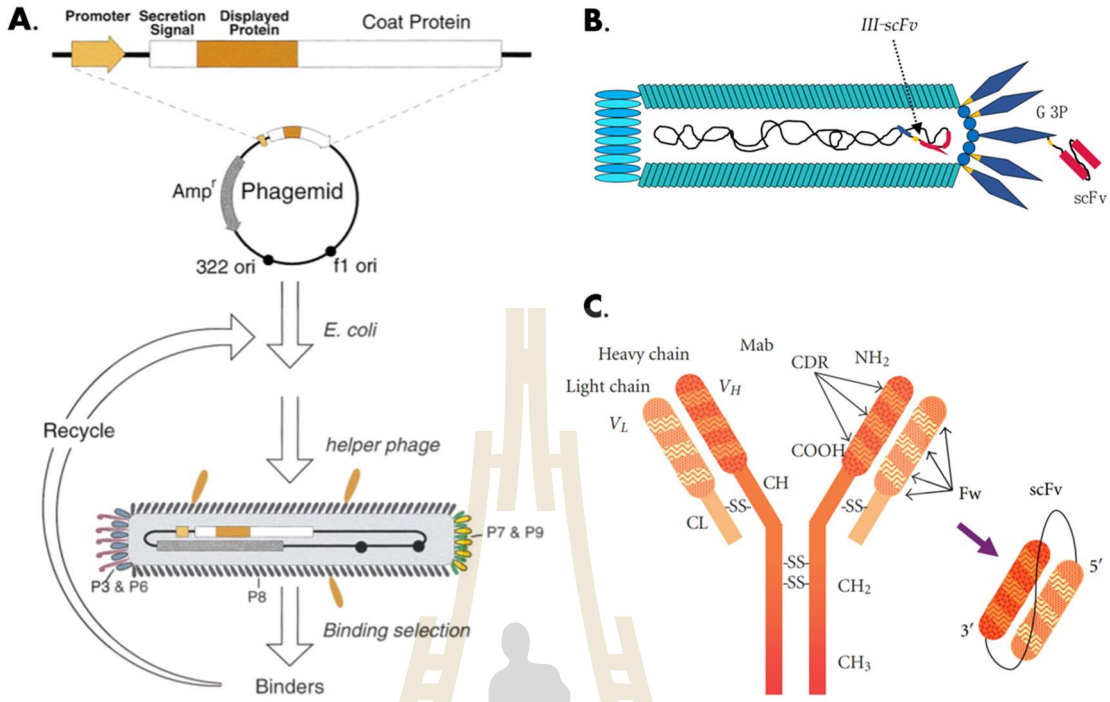

Figure 2.2 Phage display technology **A.** Phage display cycle with phagemid vectors (Sidhu, 2001). **B.** M13 phage for phage display, which engineering of the G3P is genetically fused to a human single-chain fragment (scFv) (Ledsgaard et al., 2018). **C.** Antibody format and scFv model (Ahmad et al., 2012).

2.5.3.1 Agglutination

Agglutination is the process that the antigens are linked together with corresponding antibodies. The linked maybe macroscopically or microscopically visible as clumps, aggregates, or agglutinates. Agglutination reaction depends on a firm structural relationship of exposed bacterial antigen and the antibody (Somasegaran and Hoben, 2012). Agglutination is widely used as a technique to identify specific bacterial antigens, and in turn, the identity of bacteria. It is the simply technique base on serology to use and has been widely applied in ecologic investigations and many taxonomic studies. The precipitation has been always used for work with rhizobia. The precipitation reaction occurs when soluble antigens are contacted with the corresponding antibody, in the precipitation antigens are not whole bacterial cells, but are proteins or polysaccharide molecules in solution (Somasegaran and Hoben, 2012).

2.5.3.2 Immunofluorescence (IF) or fluorescent antibody (FA) technique

Immunofluorescence (IF) or fluorescent antibody (FA) is one of the most simply, rapid, and sensitive of the serological methods that available to study rhizobia. It has been widely used for working with rhizobia, such as, used to examine identity of rhizobia strains, to identify in the nodule of bacteria, to detect nodules with double infected, to study rhizobium in soil, and make quantitative studies of rhizobium in soil (Bohlool and Schmidt, 1970). Chemical dyes such as fluorescein isothiocyanate (FITC) and lissamine rhodamine have the capacity of fluorescing when excited with UV light. Rhizobial antibodies developed in rabbits can be applied by conjugated with fluorescing chemical dyes or fluorochromes for working with rhizobia. The chemical dye commonly used for labeling the specific rhizobia antibody is FITC, which has an green fluorescence when irradiation with blue light (Somasegaran and Hoben, 2012). This technique is very popular and has been always used in study for rhizobium detection because specificity, rapid, simple, and non-genetic engineering technique (Bohlool and Schmidt, 1970). Immunofluorescence was used for detection of *B. japonicum* in soil (Bohlool and Schmidt, 1973) and for detection of *Bradyrhizobia* sp. DOA9 in pure culture and nodule (Vu et al., 2017). On the other hand, the immunofluorescence technique was used to study nodule occupancy of mixed culture inoculant and detection of rice endophytic bradyrhizobia as described by Khaing et al, (2021). However, limitation of this technique is requires fluorescent microscopy (Vu et al., 2017).

2.5.3.3 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is widely used for detection the antigens. The technique involves with immobilizing of antibody to the protein of interest within the plastic wells of a microtiter plate (Delves et al., 2017). Generally, enzyme in immunoassay, as the antibody or antigen is tagged with an enzyme (e.g., alkaline phosphatase). After completing the assay, the presence of signaling or absence of the enzyme-labeled is detected by the addition of substrate (e.g., para-nitrophenylphosphate) resulting in a colored product (Somasegaran and Hoben, 2012). This technique is very popular same as with IF technique. The main advantage is no microscopic equipment required. This technique can be used for detection of *Rhizobium* both in culture and from root nodules of soybeans and cowpeas (Asanuma et al, 1985). Moreover, ELISA technique can be used to detect rhizobia as described by Vu et al, (2017) which is potential application in agriculture, and in case of study by Khaing et al, (2021)

ELISA technique can be used to detect *Bradyrhizobium* in root of rice to study capability to endophyte in rice. It also can be used to study nodule occupied in mung bean nodule when co-inoculant 1:1. The summarize of advantages and limitations of each technique was shown in **Table 2.1.** Nevertheless, monoclonal antibody (mAb) is required for these serological techniques.

Table 2.1 Comparison of detection technique for rhizobium (Vu et al, 2017).

Detection technique for *Rhizobium*	Advantage	Limitation
Green fluorescent protein (GFP)-marked strains	It simply and substrate are not necessary, and there is no presence of background activity in indigenous soil microorganisms and plants.	Using only in the laboratory because GMO in Thailand is not acceptable, and requires fluorescent microscopy equipment.
PCR-Amplified 16S rRNA Genes	Specificity and simple.	These methods need the step of PCR product sequencing to verify each bacterial strain and thus are not appropriate for routine identification of mixed culture
Agglutination	The technique is rapidly and does not requirement of specialized equipment and skill.	Not specificity because the cross-reactivity between rhizobia is common with non--adsorbed primary antisera.
Immunofluorescence (IF)	Specificity, rapidly and simply.	Requires fluorescent microscopy equipment.
Enzyme-Linked Immunosorbent Assay (ELISA)	Specificity and rapidly, microscopic equipment is not required.	Required an enzyme and microplate reader to determine the reaction for quantitative result.

2.6 Generation of monoclonal antibody

2.6.1 Hybridoma technology

Hybridomas are engineered cells that desired to produce antibody in large amounts. Hybridoma technology was first discovered in 1975 by Georges Kohler of West Germany and Cesar Milstein of Argentina. It has been awarded the 1984 Noble prize for physiology and medicine. Normally, the cost to produce one monoclonal antibody (mAb), using the hybridoma technology is between $8,000 and $12,000. To produce monoclonal antibodies, that has been challenged with the relevant antigen because B-cells are removed from the spleen of an animal, then these B-cells are fused with myeloma tumor cells and grow in indefinitely culture (myeloma is a B-cell cancer). The fused hybrid cells (called hybridomas), being cancer cells, will multiply rapidly and indefinitely produce large amounts of the desired antibodies (Pandey, 2010).

2.6.2 Phage display technology for rhizobium detection

The phage-display is technique to isolate of directly antibodies from diverse repertoires of antibody genes. These antibody genes are fusion proteins which expressed on the surface of filamentous bacteriophages. Therefore, antibody phage-display technology permits the immortalization of monoclonal antibody genes. This technique also has capability to improve the antibody suit various applications, such as increasing of binding affinity, enhancing stability, or fusion with reporter genes. From various recombinant antibody a single-chain fragment variable (scFv) format is particularly favorable. An scFv is small molecule that consists with a variable region of heavy chains and light chains and linked together with a flexible linker. The advantages of scFv molecules are small size, long storage, and the capability to engineer and produce at large scale. Phage-display technology is a useful for the generation of scFv molecules which desired for binding affinity and specificity for many antigen types. Isolation of specific peptides and monoclonal antibodies against various targets including different pathogenic bacteria, viruses, parasites, and mycotoxins has been successfully by phage display antibody technology. In the cases of rhizobia research, phage-display technology has been used for the identification of extra-cytoplasmic proteins (Vu et al., 2017). Therefore, phage display technique was used to reduce the problem of cross-reaction occurring from polyclonal antibody or difficulty in monoclonal antibody production. Application of phage display technique as described by Vu et al., (2017) was applied for production of

monoclonal antibody using immune system of rabbit as traditional way to produce polyclonal, it decrease difficulty in monoclonal antibody production. Otherward, reported phage display technique was applied for production of monoclonal antibody which screening of specific antibody against to *Bradyrhizobium* from human phage display antibody library (Khaing et al., 2021). The procedure to screening of interest antibody using phage display technology contain with construction of phagemid **(Fig. 2.2A)** that engineer to displayed antibody, in case of Vu et al, (2017) constructed from rabbit immune system and (Khaing et al, 2021) constructed from human and used helper phage for binding selection which engineering of the G3P **(Fig. 2.2B)** is genetically fused to a human single-chain fragment (scFv) **(Fig. 2.2C).**

CHAPTER III

RESERCH METHODOLOGY

3.1 Affinity selection phage displayed scFv antibodies which are specific to each peanut *Bradyrhizobium*

To select the phage displayed scFv antibodies which are specific to each peanut *Bradyrhizobium* strain obtained from DOA, the methodologies were conducted using phage displayed technology and scFv ELISA. The experiments were performed as described below.

3.1.1 Antigen preparation

The selected peanut bradyrhizobia obtained from DOA including DASA03156, DASA03118, DASA03084, DASA03094, and DASA03028 were used for antigen preparation. To prepare the antigen, *Bradyrhizobium* was cultured in Yeast extract Mannitol Broth medium (YMB) in the incubator shaker at 28°c for 7 days. Then, the cells were washed 3 times with sterilized 0.85% (w/v) saline buffer and the cell concentration were adjusted to 10^9 cells/ml (optical density at 600_{nm} = 1) and separated into 2 groups. First group, the cell was boiled in water bath for 1 hour and stored at -20°c until use. Second group, the cell was stored at -20°c until use without boiling. Then, the total protein of antigen was measured by Bradford technique (Bradford, 1976).

3.1.2 Affinity selection of phage displayed scFv antibodies

The biopanning method was used to select phage clone that specific binding with each antigen prepared from selected peanut *Bradyrhizobium* strain. Each round of biopanning, each of antigen target (boil and non-boil) was immobilized onto immunotube with 20 µg of antigen protein in 400 µl of 100 mM $NaHCO_3$ at pH 8.5 then stored at 4 °c overnight. *Escherichia coli* TG1 was cultured on 2xYT broth and then incubated at 37 °c,

200 rpm for overnight. The 2% (w/v) skimmed milk in phosphate-buffer saline (MPBS) pH 7.4 was added into immunotube for block the nonspecific binding of phage particles, and stand at room temperature for 2 hours. Phage antibody library (Yamo Library) of 10^{12} PFU was added in immunotube. Phosphate-buffer saline (PBS) and 0.05 % (v/v) Tween 20 in phosphate-buffer saline (PBS) was used for washing step. Bounding phage was eluted by trypsin buffer and Glycine-HCl (pH 2.0) and neutralized by adding neutralization solution. *E. coli* in log phase period was infected by elute phage then spread on 2xYT plate containing 100 µg/ml ampicillin and 1 % (w/v) glucose, and then incubated plates at 37°C for overnight (Vu et al., 2017).

To continue with the next round of biopanning selection, 1 ml of 2 × YT media was added on infected *E. coli* agar plates, and then scraped surface of agar plate. The scraped cells were kept in 15% (v/v) glycerol stock at 70°C, and 10 µl of scraped bacteria was added into 10 ml of 2 × YT supplemented with 100 µg/ml ampicillin and 1% (w/v) glucose, and incubated at 37°C with shaking for 2 hours. After this procedure, 5 × 10^{10} helper phage KM13 was added and incubated at 37 °C, without shaking, for 30 minutes. Afterward, the culture medium was exchanged by centrifugation at 4,000 rpm at 4°C for 15 minutes, the supernatant was removed, and the pelleted bacteria was resuspended in 5 ml of 2 × YT containing 100 µg/ml ampicillin, 50 µg/ml kanamycin, and 0.1% (w/v) glucose. Then, it was incubated at 30°C with shaking for overnight. On the following day, the overnight culture was centrifuged at 4,000 rpm and 4°C for 15 minutes. Phage was precipitated by adding 1 ml of PEG/NaCl (20% (v/v) polyethylene glycol 6000 in 2.5 M NaCl) into 4 ml of the supernatant and kept on ice for 1 h, and later centrifuged at 4,000 rpm, at 4°C for 30 minutes. The supernatant was removed and the pellet was resuspended in 100 µl PBS for the next round of selection. The selected phage scFv clones after 4 round of bio-panning was verified by scFv ELISA (Vu et al., 2017).

3.1.3 Verification of selected phage displayed scFv antibodies which are specific to each peanut *Bradyrhizobium*

Infected *E. coli* was used for preparation of single-chain variable fragment (scFv) using 1 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG) (AppliChem, Italy) as inducer to express single-chain variable fragment (scFv) from infected *E. coli*.

Both boil and non-boil target antigens was immobilized on 96-well flat bottom ELISA maxisorp plate with 5 µg target protein dissolved in 100 µl of 100 mM $NaHCO_3$ (pH

8.5) per well. One% (w/v) Bovine Serum Albumin (BSA) in phosphate-buffer saline or 2% (w/v) skimmed milk in phosphate-buffer saline (MPBS) was used for negative control antigen. Immobilized plate was incubated at 4°C for overnight. BSA 1% (w/v) in phosphate-buffer saline or 2% (w/v) skimmed milk in phosphate-buffer saline (MPBS) was added into well plate for blocking nonspecific binding of phage particles then phosphate-buffer saline (PBS) and 0.05 % (v/v) Tween 20 in phosphate-buffer saline (PBST) was used in washing step. His-probe horseradish peroxidase (HRP) Conjugate (ThermoFisher, USA) in PBS (1:5,000) was added for detected bond scFv. Color reactions was developed using ABTS (2, 2-azino-di-3-ethyl-benzothiazoline6-sulfonate) peroxidase substrate containing 0.05% H_2O_2. Detection was done by measuring the absorbance at 405 nm in an ELISA plate reader. Selected scFv which are specific to each *Bradyrhizobium* strain provided by DOA was further used to produce soluble scFv antibody in overexpressed host condition and checked cross-reactivity with other *Bradyrhizobium* strains (Vu et al., 2017).

3.2 Production of soluble scFv antibody

Genomic DNA (gDNA) were extracted from meat and root hair samples by DNA GF-1 Blood DNA Extraction kit (VIVANTIS, Malaysia). The DNA quality and yield were checked by Nanodrop spectrophotometer (Thermo Scientific, USA). The gDNA from each animal was stored at - 20˚C until genotyping determination.

3.2.1 Cloning of scFv antibody

The plasmid of selected phagemid on pMod1 vector (clone F11 specific to *Bradyrhizobium* sp. DASA03028) was extracted using The FavorPrep™ Plasmid Extraction Kit (FAVORGEN, Taiwan). The gene of scFv antibody (clone F11) was inserted into multiple cloning sites of pET21d (+) vector between *Nco*I and *Not*I **(Fig. 3.1)** The pET21d (+) vector and scFv DNA fragment were digested with the *Nco*I and *Not*I restriction enzymes (20 U/µl, NEB, USA). The reaction for digestion of scFv antibody DNA fragment and pET21d (+) vector was performed in the total volume of 50 µl. The master-mixed for digestion reaction consist with 2 µg of DNA insert and vector DNA, 1x Cutsmart buffer, *Nco*I and *Not*I restriction enzyme (20U/µl) and water. The reactions were incubated for 16 hours at 37°C. The digested vector DNA was dephosphorylated with adding 1 µl of CIP enzyme (20 U/µl, NEB, USA) and incubated for 1 hour at 37°C. The insert and plasmid vector fragments were separated using gel electrophoresis and purified by Wizard clean up kit (Promega, USA). The reaction for ligation of scFv antibody DNA fragment and pET21d (+) vector were done

in ratio 3:1 with T4 Ligase enzyme and incubated for 16 hours at 16°C. The ligation was transformed into *E. coli* DH5α by heat-shock transformation (Khaing et al, 2021).

The F11/pET21d+ plasmid was extracted and sequencing. The sequence of F11 in pET21d+ was aligned and compared with sequence from F11 phagemid using SnapGene software.

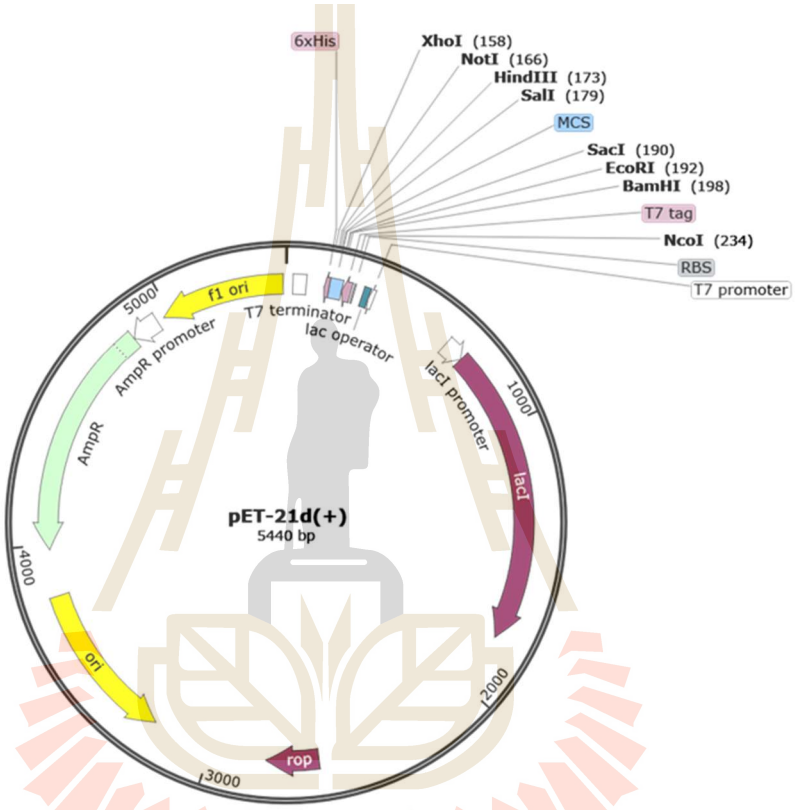

Figure 3.1 Map of pET21d+ vector. Showing of multiple cloning sites, T7 promoter, 6xHIS tag, and *ampicillin resistance* gene (Khaing et al, 2021).

3.2.2 Expression of scFv antibody

To produce soluble scFv antibody (from clone F11). The F11/pET21d+ plasmid was extracted and transformed into SHuffle® T7 competent *E. coli* (NEB, USA) and cultured on LB agar plate containing 100 µg/ml of ampicillin and incubated for overnight at 37°C. Then, the single colony was picked into 5 ml of LB containing 100 µg/ml of ampicillin and incubated with shaking (250 rpm) for overnight at 30°C as a starter. The starter was added into 400 ml of LB containing 100 µg/ml of ampicillin in flask 2-L and

incubated with shaking (250 rpm) at 30°C until the OD_{600} become 1.0, then induced by adding 1.0 mM IPTG at 16°C for 20 hr.

Collection and purification of recombinant antibody was done by centrifugation the induced *E. coli* culture at 6000 × g for 10 min, the cell pellets were resuspended in 30 ml of lysis buffer (20 mM Tris-HCl, 500 mM NaCl, and 20 mM imidazole, pH 7.4) containing 1 mg/ml of lysozyme (Thermo Scientific, USA) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Thermo Scientific, USA). Cells were sonicated for 3 times with amplitude 30%, 5 min on ice using 2 sec pulse and 2 sec pauses for cooldown sample, then soluble scFv was collected by centrifugation at 4 °C for 15 min at 8000 × g, and the supernatant was kept. The soluble protein form supernatant and inclusion bodies from cell pellet which denature by urea and determine present of protein using Western blot analysis.

The soluble scFv from supernatant was purified using 1 ml of His-Trap column (GE Health care, USA), then equilibration with binding buffer (20 mM Tris-HCl, 500 mM NaCl, and 20 mM imidazole, pH 7.4), then washed 3 times with washing buffer (20 mM Tris-HCl, 500 mM NaCl, and 60 mM imidazole, pH 7.4) and eluted with 20 mM Tris-HCl, 500 mM NaCl, and 250 mM imidazole, pH 7.4. The samples were applied for buffer exchange using snake-skin dialysis bag with 1×PBS buffer for 100,000-fold and observed the purity of protein by SDS-PAGE. The sample were collected and kept at 4 °C until use. In this cases, yiN92-e10 recombinant antibody was produced as described by Khaing et al, (2021).

The three-dimensional structure was predicted using the Phyre program at the http://www.sbg.bio.ic.ac.uk/phyre2 site.

3.3 Determination the cross-reactivity of recombinant scFv with other Bradyrhizobium strains

The ELISA technique was used to determine cross-reactivity of selected scFv with other 25 peanut bradyrhizobia strains **(Table 3.1)**. Each *Bradyrhizobium* strain was cultured in Yeast extract Mannitol Broth (YMB) in the incubator shaker for 7 days then preparation for antigens as described previously and BOX-PCR was used to differentiate isolates of the bradyrhizobia using BOX-A1R primer (5'-CTACGGCAAGGCGACGCTGACG- 3') (Figure A1) according to the method described by Phimphong et al., (2023). Each antigen was duplicate immobilized for 5 µg/well on ELISA plate, then the ELISA procedure was done as described previously with 20 µg/ml of antibody.

Table 3.1 *Bradyrhizobium* strains used for determination the cross-reactivity of recombinant scFv antibody

Strains	Geographical origin	Host origin	Source or Reference
SUTN9-2	Thailand	*A. americana*	(Noisangiam et al., 2012)
DASA03028	Thailand	*A. hypogaea*	DOA
DASA03123	Thailand	*A. hypogaea*	DOA
DASA03131	Thailand	*A. hypogaea*	DOA
DASA03120	Thailand	*A. hypogaea*	DOA
DASA03129	Thailand	*A. hypogaea*	DOA
DASA03007	Thailand	*A. hypogaea*	DOA
DASA03051	Thailand	*A. hypogaea*	DOA
DASA03054	Thailand	*A. hypogaea*	DOA
DASA03084	Thailand	*A. hypogaea*	DOA
DASA03071	Thailand	*A. hypogaea*	DOA
DASA03136	Thailand	*A. hypogaea*	DOA
DASA03112	Thailand	*A. hypogaea*	DOA
DASA03076	Thailand	*A. hypogaea*	DOA
DASA03017	Thailand	*A. hypogaea*	DOA
DASA03070	Thailand	*A. hypogaea*	DOA
DASA03061	Thailand	*A. hypogaea*	DOA
DASA03030	Thailand	*A. hypogaea*	DOA
DASA03210	Thailand	*A. hypogaea*	DOA
DASA03239	Thailand	*A. hypogaea*	DOA
DASA03177	Thailand	*A. hypogaea*	DOA
DASA03181	Thailand	*A. hypogaea*	DOA
DASA03172	Thailand	*A. hypogaea*	DOA
DASA03216	Thailand	*A. hypogaea*	DOA
DASA03050	Thailand	*A. hypogaea*	DOA
DASA03211	Thailand	*A. hypogaea*	DOA
DASA03214	Thailand	*A. hypogaea*	DOA

3.4 Checkerboard Titration (CBT)

To determine the limitation of recombinant scFv antibody on target binding, the ELISA plate was immobilized with 150 µl/well of the serial dilution of non-boil bacterial cell suspension which had the highest concentration at 10^9 cells/ml in rows A to lowest concentration at 10^4 cells/ml in rows F. The ELISA procedure was done as described previously, then the ELISA well plate was incubated with 5 different concentrations of yi028-F11 scFv in duplicate from the highest concentration in column 9 and 10 to lowest in column 1 and 2 at concentration of 0.5, 1, 5, 10, 20 µg/ml, respectively.

3.5 Investigation the influence of bacterial aging on scFv binding affinity

To optimize affinity between scFv antibody in relation to the cell age of bradyrhizobial antigen, the ELISA plate was immobilized with non- boil bacterial cell of (i) colony culture on agar plate at age of 5, 7, 15, 30 days after incubation, then the colony was dissolved with 100 mM sodium bicarbonate and adjusted the OD 600_{nm} to 0.2, and (ii) broth culture at age of 5, 7, 15, 30 days after inoculation, then the cell concentration was adjusted the OD 600_{nm} to 1 using 100 mM sodium bicarbonate. Afterward, the ELISA was performed as described previously with the concentration of scFv antibody at 20 µg/ml.

3.6 Determination the viable cell number of each Bradyrhizobium strain in single- and mixed-culture inoculant using Fluorescence Antibody (FA) technique

The experiment was designed to determine the number of viable cell from both liquid- and peat-base inoculants. The liquid inoculant was prepared as same as DOA. Each *Bradyrhizobium* strain was prepared for a starter as described previously, the liquid inoculant was prepared from inoculation of 1% starter to yeast extract mannitol (YM) containing with 2% Polyvinylpyrrolidone (PVP) and storage for 12 months (6 months were kept at room temperature and another 6 months were kept at 4°C). The cells were collected and 0, 1, and 12 months. The peat-base inoculant was prepared using peat-base from DOA, the peat was neutralized to the final pH7 and packed 90g of peat in polypropylene bag (18×28.5 cm^2) and inoculated with *Bradyrhizobium* to obtain the final cell at 10^8cells/g of peat, then incubated at room temperature for 1 week.

Determination of the cell number of each living *Bradyrhizobium* strain in liquid inoculant was done by FA technique using specific scFv yi028-F11 against *Bradyrhizobium* sp. DASA3028, and using specific scFv yiN92-e10 against *Bradyrhizobium* sp. SUTN9-2 (Khaing et al, 2021). The viable cell count was performed in both single- and mixed-culture inoculant (*Bradyrhizobium* sp. DASA3028 + *Bradyrhizobium* sp. SUTN9-2 in ratio 1:1). To determine the number of viable cell, the inoculant was 10-fold diluted using 0.85% (w/v) NaCl as diluent and spread on YEM ager media for total plate count. The culture medium plate that had colony in range of 30-300 was picked up to do FA technique. Then, each single colony was picked, smeared and heat fixed on glass slide, then the specific antibody was dropped for 1 µg on smeared colony and incubated at room temperature for 20 min. The smeared was washed 3 times with 1xPBS pH7.4 and covered with 1:500 of anti-His Dylight 488 (ThermoFisher, USA) secondary antibody in 1xPBS pH7.4 for 20 min at room temperature, then washed 3 times with 1xPBS and observe under fluorescence microscope. The number of each bradyrhizobial cell was estimated from the positive signal of FA in relation to the number of cell from total plate count technique. The schematic of experimental procedure was shown in **Fig. 3.2.**

The number of colonies forming unit (CFU) of individual strain was calculated as:

$$\text{CFU/ml or CFU/g of DASA03028} = \frac{A}{0.1\ (mL) * Dilution\ factor}$$

$$\text{CFU/ml or CFU/g of SUTN9-2} = \frac{B}{0.1\ (mL) * Dilution\ factor}$$

$$\text{CFU/ml or CFU/g of contaminant} = \frac{C}{0.1\ (mL) * Dilution\ factor}$$

$$\text{CFU/ml or CFU/g of total colony on plate} = \frac{A+B+C}{0.1\ (mL) * Dilution\ factor}$$

A = No. of positive colony after stained with yi028-F11

B = No. of positive colony after stained with yiN92-e10

C = No. of non-signal colony after stained with both antibodies

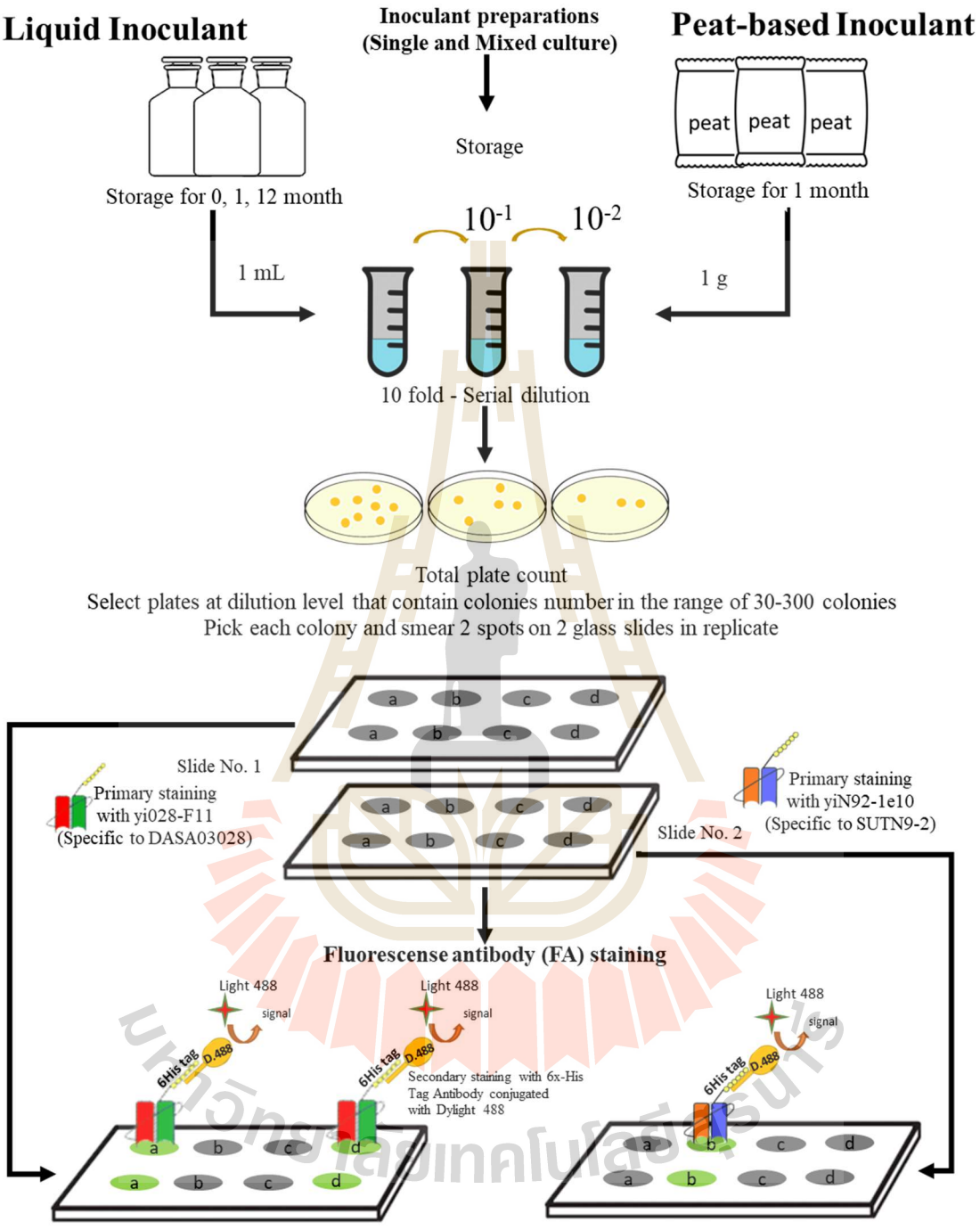

Figure 3.2 Schematic of procedure for Determination of the viable cell number of *Bradyrhizobium* strain in liquid- and peat- base inoculant by FA technique.

3.7 Detection of of Bradyrhizobium bacteroid in the nodule

Under the laboratory experiment, the seeds of peanut (cultivar Tainan 9 (TN9), Kalasin 2 (KS 2), Songkhla 2 (SK2), Khonkaen 5 (KK5)) and mung bean (cultivar Suranaree 1

(SUT1), Suranaree 4 (SUT4), Chainat 72 (CN72) and Kampangsean 2 (KPS2)) were sterilized. In case of peanut, the seeds were soaked in 95% ethanol for 30 seconds. Then, all seeds were washed with sterilized water five times and soaked in 3% (v/v) sodium hypochlorite for 30 second and washed with sterilized water 5-6 times to remove sodium hypochlorite. In case of mung bean, the seeds were sterilized in the same step as peanut except soaking in 3% (v/v) sodium hypochlorite for 5 minutes. Then, all seeds were soaked in sterilized water overnight at room temperature under dark condition. Then, peanut seeds were germinated on sterilized vermiculite for 3 days, while mung bean seeds were germinated on 0.8% (w/v) sterilized water agar plate in the dark conditions at room temperature. The germinated seeds were planted in Leonard's jars and inoculated with *Bradyrhizobium* at a total of 10^9 cells/seed of each treatment. In case of mixed culture, the numbers of *Bradyrhizobium* sp. DASA03028 and SUTN9-2 cell were adjusted according to the ratio of 1:1. All plants were supplemented with N-free medium (Somasegaran and Hoben, 2012) and grown in light room for 1 month which provided a flux density of light of about 450/μEs^{-1} m^{-2} with a 12–12 h light–dark regime. After planting for 1 month, the nodules in each experiment were collected and crushed in 100 mM sodium carbonate buffer pH 8.5. The bacteroid suspension was centrifuged at 6,000 rpm for 1 minute to precipitate plant tissue, then 150 µl of bacteroid suspension was immobilized in the each well. Then, the specific scFv for each *Bradyrhizobium* was used to perform ELISA technique as described previously.

The FA technique was also used to observe the present of bacteroid in the nodule. The FA procedure was done as described by Khaing et al, (2021), The stained nodule was observed under a Confocal laser scanning microscope (Nikon A1, Japan) and exhibited green fluorescence when excited by a blue laser at 488 nm.

The influence of bacteroid from different nodules in veriety levels of peanut namely Tainan 9 (TN9), Kalasin 2 (KS 2), Songkhla 2 (SK2), Khonkaen 5 (KK5) and mung bean i.e., Suranaree 1 (SUT1), Suranaree 4 (SUT4), Chainat 72 (CN72), and Kampangsean 2 (KPS2) to the binding between antigen and antibody was observed using ELASA technique as described previously.

CHAPTER IV

RESULTS

4.1 Affinity selection phage displayed scFv antibodies

The biopanning procedure was performed for affinity selection of each peanut *Bradyrhizobium* sp. using naïve human library (Yamo library) (Pansri et al., 2009). **Table 4.1** provides a summary of the affinity selection process during biopanning against five distinct peanut bradyrhizobia strains.

Table 4.1 Summary during the biopanning process of affinity selection results against peanut *Bradyrhizobium* strains

Affinity selection step	*Bradyrhizobium* sp.				
	DASA03156	DASA03118	DASA03084	DASA03094	DASA03028
Rounds of selection	4	4	4	4	4
Colonies obtained (CFU/ml)	3.81×10^6	8.77×10^4	2.34×10^6	9.93×10^5	3.74×10^6
Colonies pickup	16	16	16	16	16
Positive clones at monoclonal phage ELISA	0	0	0	0	1 (yi028-F11)

After the 4th round of biopanning $3.81x10^6$ clones against DASA03156, $8.77x10^4$ clones against DASA03118, $2.34x10^6$ clones against DASA03084, $9.93x10^5$ clones against DASA03094, and $3.74x10^6$ clones against DASA03028, the 16 clones against each peanut *Bradyrhizobium* were randomly picked up and verified their affinity by phage ELISA. Among these clones, one positive clone (yi028-F11) exhibited an absorbent 405nm (A_{405nm}) value against the strain DASA03028 more than two-fold higher than that of negative control (2% skim milk) and this clone was selected for further studies **(Fig. 4.1A)**. In contrast, the positive clones against other bradyrhizobia resulted in less than a two-fold increase in OD. Importantly the selected phage clone yi028-F11 displayed no cross-reactivity with other bradyrhizobia and *Pseudomonas aeruginosa* **(Fig. 4.1B)**.

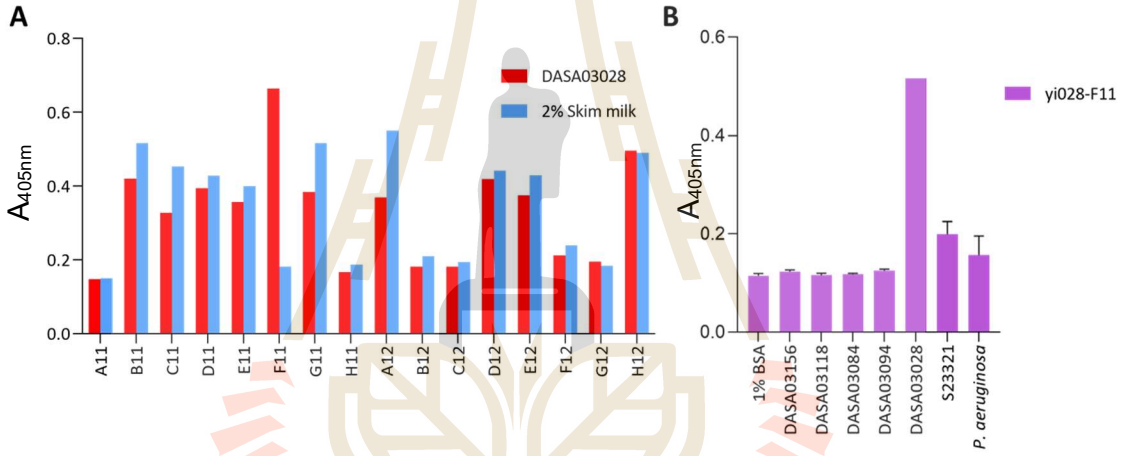

Figure 4.1 Verification of selected phage clones by phage ELISA. (A) Phage ELISA of scFv clones after 4th round of biopanning binding to peanut *Bradyrhizobium* strain DASA03028 (red bar) compared with 2% skim milk (blue bar); (B) Phage ELISA binding of scFv antibody clone yi028-F11 against *Bradyrhizobium* strain DASA03028 and other bradyrhizobia and *Pseudomonas aeruginosa*, using 5 µg of boiled liquid culture as antigen. Values are the mean of duplicate wells. Error bars show the standard deviation for each set of data

Given that *Bradyrhizobium* sp. SUTN9-2 was employed with strain DASA03028 as the dual-culture inoculant model in this study, we conducted an evaluation of the cross-reactivity of specific phage clones with each bacterium. In particular, we re-examined clone yiN92-e10, which is specific to strain SUTN9-2 (Khaing et al, 2021), and clone yi028-F11, specific to DASA03028, for potential cross-reactivity against both bacteria in two forms: boiled and non-boiled cells. Our results revealed no cross-reactivity between these clones and both forms of antigens, as depicted in **Fig. 4.2**. Notably, clone yiN92-e10 exhibited a stronger affinity for strain SUTN9-2 compared to clone yi028-F11's affinity for DASA03028.

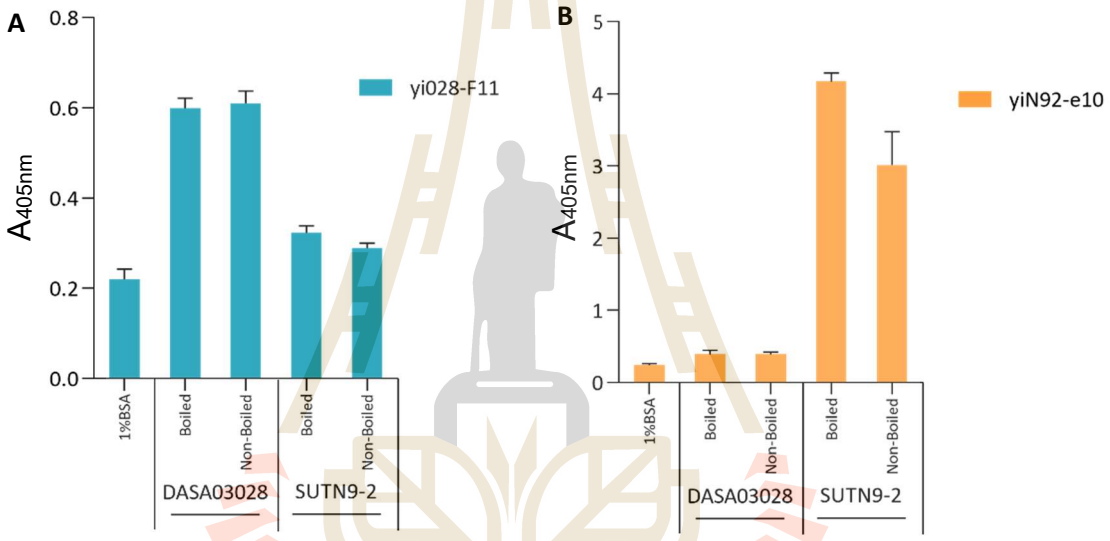

Figure 4.2 Specific binding of selected phage-displayed scFv clones yi028-F11 (A) and yiN92-e10 (B) against 5 µg of bolied and non-boiled cell antigens *Bradyrhizobium* sp. DASA03028 and *Bradyrhizobium* sp. SUTN9-2 by ELISA. Values are the mean of duplicate wells. Error bars show the standard deviation for each set of data.

4.2 Production of soluble scFv antibody

To produce more quantity of antibody in *Escherichia coli* SHuffle T7 (C3029), the recombinant scFv antibody was constructed. The phagemid vector from clone yi028-F11 was extracted and the gene fragment encoded for scFv was digested with *Nco*I and *Not*I restriction enzymes. Subsequently, this fragment was inserted into pET21d (+) vector as the recombinant plasmid **(Fig. 4.3A)** and expressed in an *E.coli* host that promotes disulfide bond formation in the cytosol (Ren et al., 2016). The three-dimensional structure

prediction of scFv antibody using Phyre^2 program is shown in **Fig. 4.3B**. The amino acid sequence of scFv fragment and complementarity-determining region (CDR1, CDR2, and CDR3) of the VH and VL is shown in **Fig. 4.3C**. The production of scFv antibody was expressed by the induction with isopropyl β- d-1-thiogalactopyranoside (IPTG). After induction, cell pellet was lysed using sonicator for ultrasonic disruption. The supernatant and cell pellet were examined using Western blot analysis to observe and verify the expressed protein in soluble and inclusion bodies **(Fig. 4.3D)**. The soluble scFv was collected and purified using His-Trap column for affinity chromatography procedure. The purity protein band was observed in elution fraction at the expected molecular weight of approximately 28 kDa on SDS-PAGE gel **(Fig. 4.3E)**. The soluble scFv antibody of yiN92-e10 was also produced in *E. coli* as described by Khaing et al, (2021).

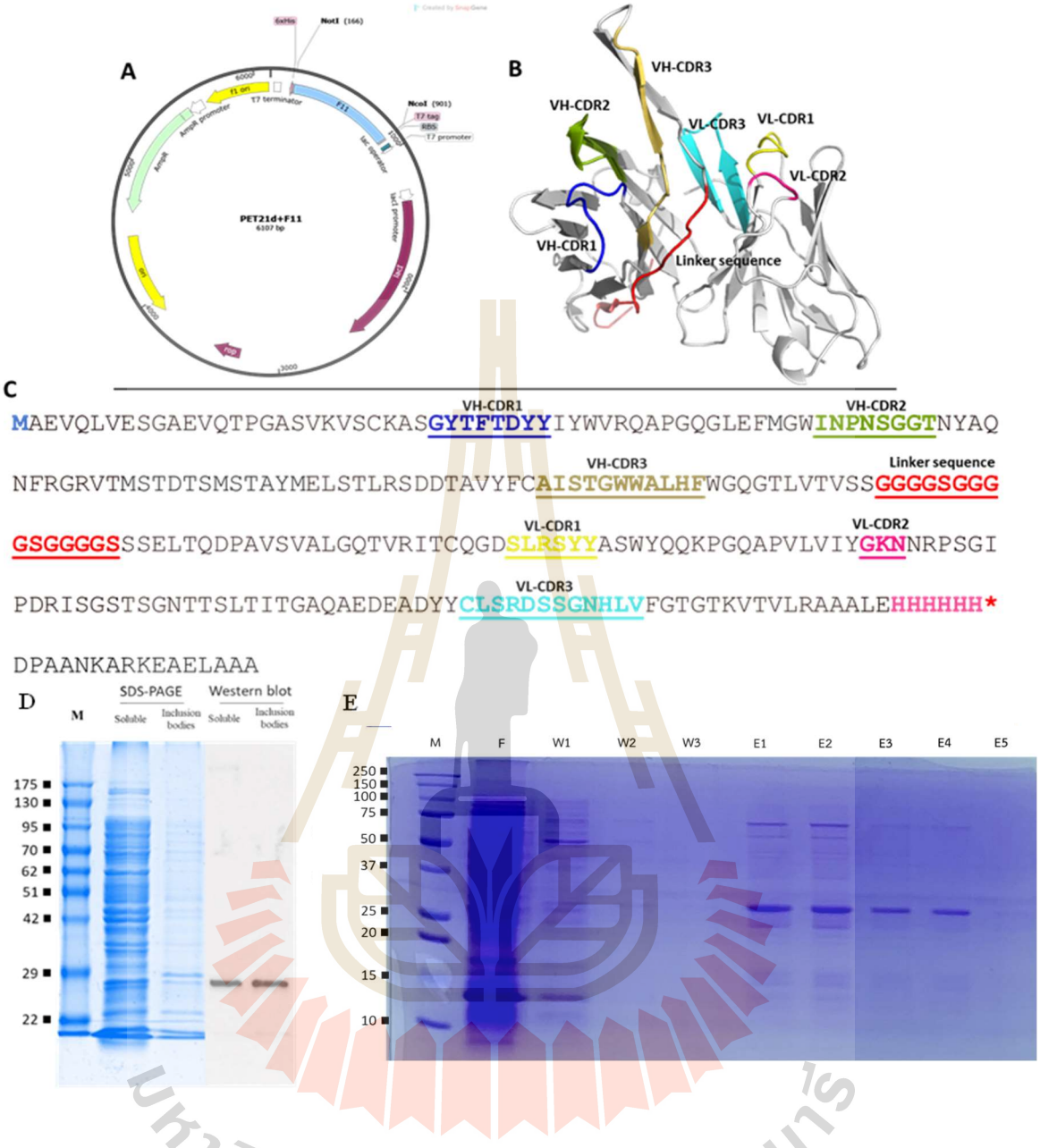

Figure 4.3 (A) Map of pET21d+/F11 plasmids. The scFv fragments were subcloned into pET-21d (+) vector between *Nco*I and *Not*I sites. (B) Three-dimensional structure of scFv antibody yi028-F11. (C) Amino acid sequence analysis of soluble scFv clone yi028-F11 on pET21d+/F11 plasmids indicated the sequence of complementary determining regions (CDR), VH and VL chains and linker were labeled in underlines. (D) 12% SDS-PAGE and Western blot analysis of scFv F11 antibody presented in soluble and inclusion bodies. The soluble F11 scFv antibody against *Bradyrhizobium* sp. strain DASA03028, Lane M: protein molecular weight marker, Lane 2: total protein from soluble (supernatant), Lane 3: total protein from inclusion bodies (denatured with 6M

urea). (E) 12% SDS-PAGE analysis of purified soluble scFv yi028-F11 antibody. Lane M, protein molecular weight marker; lane F, flow through fraction; lane W1, W2, W3 are washed fraction; lane E1, E2, E3, E4 and E5 are the five fractions of elution. The molecular weight of soluble scFv is approximately 28 kDa can be found in elution fractions E1 to E4, all eluted fractions were dialyzed for buffer exchange.

4.3 Optimization of antibody-bacterial cell concentration and determination of cross-reactivity with other peanut Bradyrhizobium strain

To determine the optimum concentration of antibody and the target bacterium antigen, the ELISA technique with a checkerboard titration was performed. The results showed that the highest concentration of yi028-F11 scFv at 20 µg/ml could detect $1.0x10^4$ cells/ml of non-boiled bacterial antigen. Lowering the antibody concentration in range of 5-10 µg/ml could detect the antigen only when the cell concentration more than $1.0x10^9$ cells/ml, while the lowest scFv concentration at 1 µg/ml could not efficiently detect although using the highest concentration of target bacteria at $1.0x10^9$ cells/ml **(Fig. 4.4)**. These results concluded that the optimal ELISA condition is $1.0x10^9$ cells/ml ($1.0x10^8$ cells/well) of antigen with scFv concentration at 20 µg/ml.

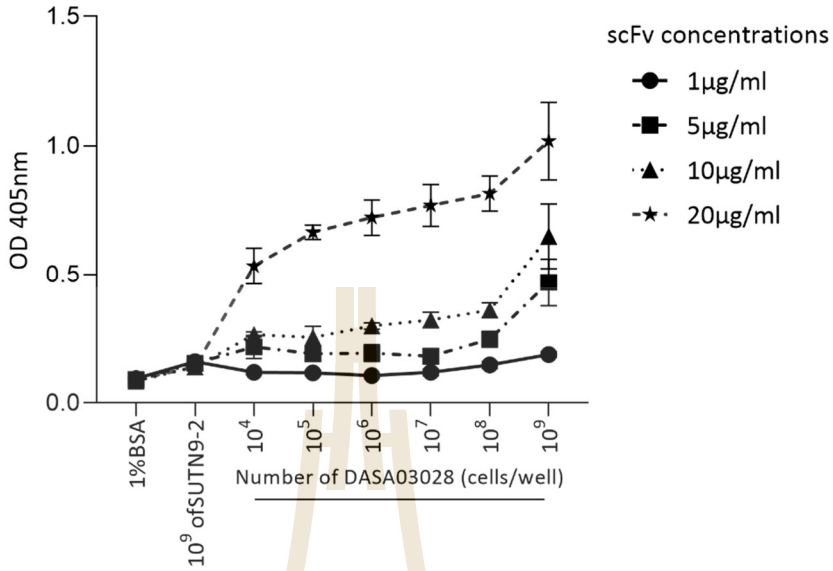

Figure 4.4 Checkerboard titration analysis against non-boiled whole cell bacteria. The detection limit of the recombinant yi028-F11 scFv antibody on *Bradyrhizobium* sp. DASA03028 was examined by ELISA, using a dilution series of recombinant yi028-F11 scFv antibody concentration and serial dilutions of bacterial concentration as indicated. 1% BSA and 10^9 cells/ml of non-boiled *Bradyrhizobium* sp. SUTN9-2 were used as negative control. The lines are presented the average absorbance values of triplicate samples and the error bars represented the standard error of average.

The product of recombinant yi028-F11 scFv antibody was further confirmed its binding with *Bradyrhizobium* sp. DASA03028 and rechecked the cross-reactivity against 25 strains non-boiled form antigen of other peanut bradyrhizobia using ELISA. Based on the result of non cross-reactivity, it confirms the binding and the specificity of this recombinant scFv antibody on the strain DASA03028 **(Fig. 4.5)**.

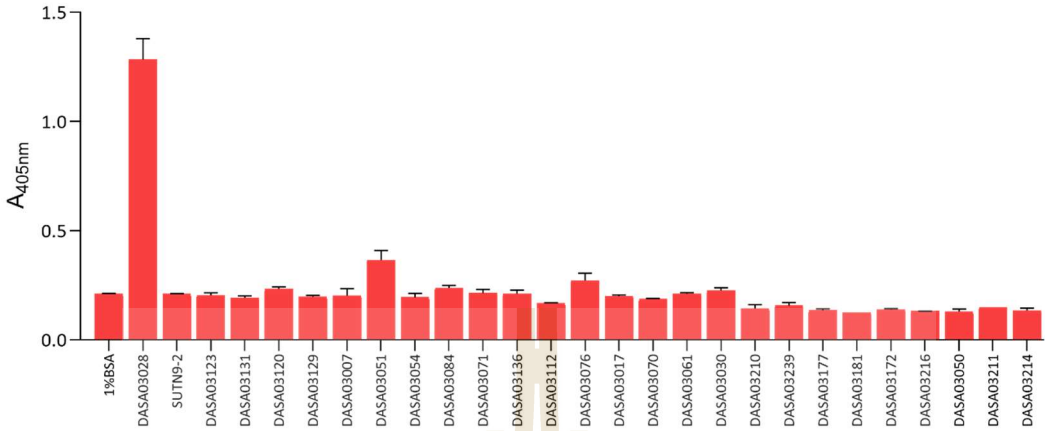

Figure 4.5 The cross-reactivity determination of the recombinant yi028-F11 scFv antibody to non-boiled target antigen of 25 strains peanut bradyrhizobia. Values are the mean of duplicate wells. Error bars show the standard deviation for each set of data.

4.4 The influence of bacterial cell aging on antibody binding ability

The age of antigen cells can significantly impact the binding of recombinant scFv antibodies. To explore this, the binding of antibodies against different cell ages of strain SUTN9-2 and DASA03028 grown in both solid medium (colony form) and liquid culture media at various time points: 5, 7, 15, and 30 days after inoculation (dai) using ELISA. For DASA03028, the recombinant yi028-F11 scFv antibody exhibited stronger binding with cells from liquid culture than with those from colony-forming on solid medium. The highest signal was observed at 7 dai for the colony-forming cells and at 30 dai for liquid culture cells. However, cells from colonies aged between 5 and 30 days were still detectable using the ELISA technique **(Fig. 4.6A and 4.6C)**. In contrast, the recombinant yiN92-e10 scFv antibody, specific to strain SUTN9-2, displayed better binding to colony culture compared to liquid culture. Colony-formed cells of varying ages, from 5 to 30 days, exhibited similar binding efficiency with the scFv antibody. The cells from liquid culture of strain SUTN9-2 at 15 dai produced the highest signal. Remarkably, the signal remained

consistently more than two-fold higher than that of the non-target antigen (1% BSA), even when the cell age was either younger or older than 15 dai **(Fig. 4.6B and 4.6D)**.

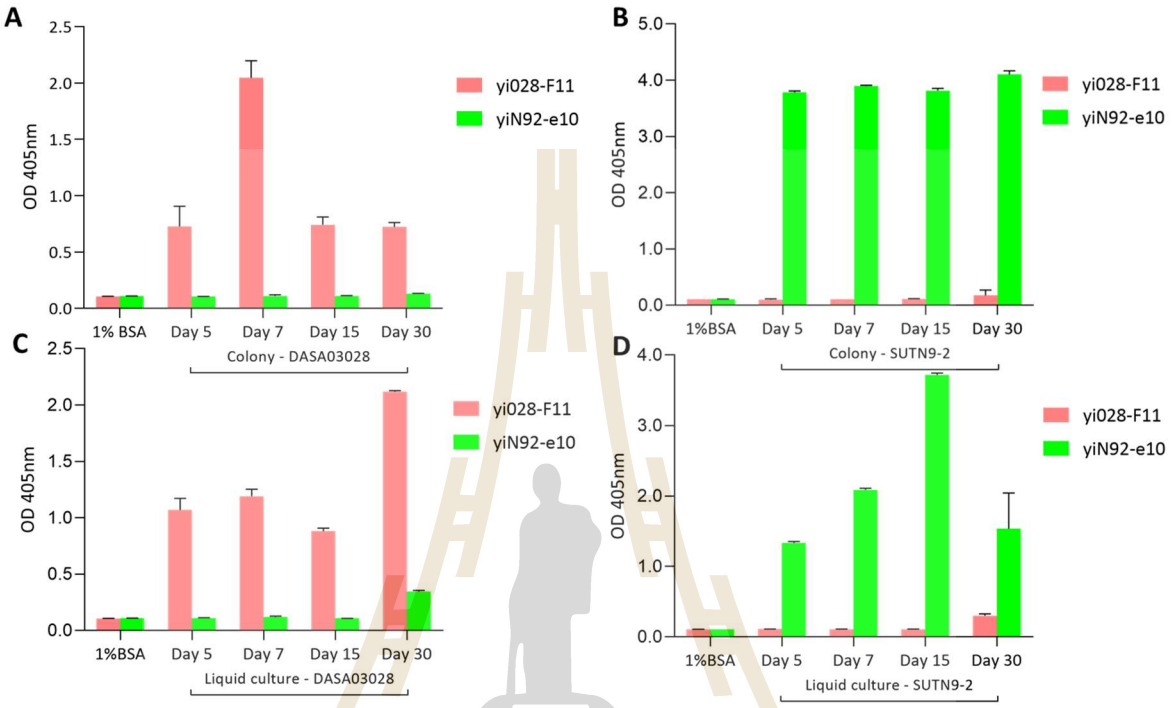

Figure 4.6 The influence of antigen cell age was determined using ELISA. The binding of soluble scFv yi028-F11 antibody (pink bar) and yiN92-e10 (green bar) which specific for *Bradyrhizobium* sp. DASA03028 and SUTN9-2, respectively on the colony age of (A) DASA03028 and (B) SUTN9-2 at 5, 7, 15 and 30 days after incubation on YMA agar plate, and on the liquid culture of (C) DASA03028 and (D) SUTN9-2 at 5, 7, 15 and 30 days after inoculation in YMB medium. BSA was used as non-target antigen. Values are the mean of triplicate wells. Error bars show the standard deviation for each set of data.

4.5 Application the recombinant scFv antibodies for determination the viable cell counts of individual bradyrhizobium in the mixed-culture liquid- and peat-based inoculant through the implement of fluorescent antibody (FA) technique.

This technique aims to confirm the presence of individual colonies on agar plates following the total plate count conducted using the FA technique. It involves the identification of viable cells through the application of their respective scFv antibodies, allowing for the quantification of each strain's cell count within the inoculant. An appropriate dilution level was chosen, ensuring a colony count within the range of 30-300 colonies. The results obtained through the FA technique are illustrated in **Fig. 4.7**. Positive colonies, which exhibit specificity to the antibody, emitted a vivid green fluorescent color, while non-specific colonies displayed no fluorescent signal under the fluorescent microscope.

Figure 4.7 Fluorescence antibody (FA) of scFv antibodies yi028-F11 (A-D) and yiN92-e10 (E-H) tested on *Bradyrhizobium* sp. DASA03028 (A-B and E-F) and SUTN9-2 (C-D and G-H) on glass slide for FA assay under fluorescent microscope. The result of positive (B and H) and negative (D and F) samples were observed under fluorescent light, while bacteria smear observing without fluorescent light were also demonstrated (A, C, E, and G)

Liquid inoculant

This technique was employed to assess viable cell counts in both single- and mixed-culture liquid inoculants at various storage durations. In the case of the single-culture inoculant (**Fig. 4.8A**), the initial cell count for DASA03028 was $9.4x10^6$ CFU/ml, which subsequently increased to $3.3x10^8$ CFU/ml after 1 month and reached $9.3x10^8$ CFU/ml after 12 months of storage. Meanwhile, for SUTN9-2 in the liquid inoculant, FA analysis determined cell counts of $7.2x10^6$, $5.2x10^8$, and $9.5x10^7$ CFU/ml after 0, 1, and 12 months of storage, respectively (**Fig. 4.8A**).

In the mixed liquid culture inoculant, comprising both strains at a 1:1 ratio (**Fig. 4.8B**), FA analysis indicated the following viable cell counts: for DASA03028, $4x10^6$ CFU/ml at the beginning, $9.9x10^7$ CFU/ml after 1 month, and $5.6x10^8$ CFU/ml after 12 months of storage (**Fig. 4.8B**). Remarkably, in the mixed-culture liquid inoculant, SUTN9-2 initiated at $3.5x10^6$ CFU/ml, then decreased to $2x10^6$ CFU/ml after 1 month of storage, and remained at $7x10^7$ CFU/ml after 12 months (**Fig. 4.8B**). Importantly, based on the FA technique, no contaminants were detected in the inoculant.

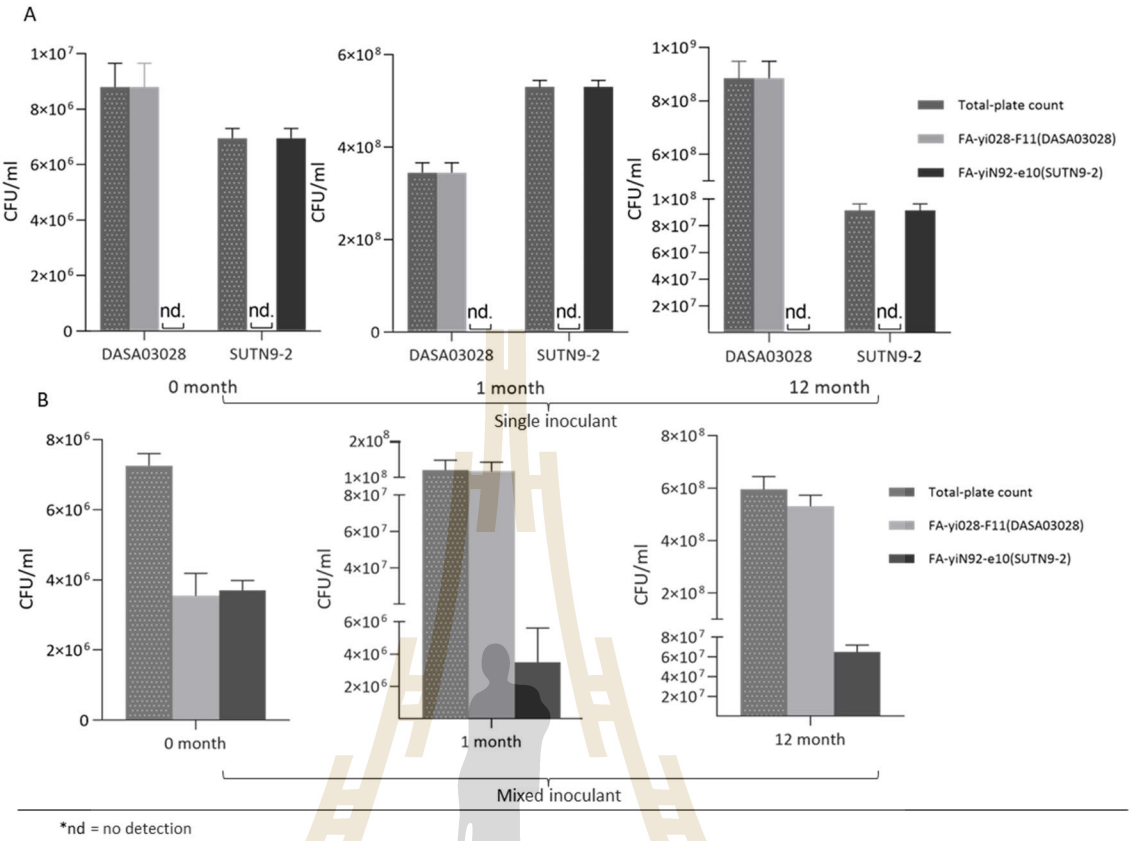

Figure 4.8 Determination the cell number of *Bradyrhizobium* sp. DASA03028 and SUTN9-2 in (A) single and (B) mixed-culture liquid inoculant using FA technique at different times after storage.

Peat-based inoculant

This technique was further applied to assess peat-based inoculants, a known source of potential contaminants. Initially, each bradyrhizobium was introduced into the inoculant at a concentration of 10^8 cells/g. Subsequently, the technique revealed that in single-culture peat-based inoculants, DASA03028 exhibited a viable cell count of $5.5x10^8$ CFU/g at 0 month, for strain SUTN9-2, the viable cell count was $7.1x10^8$ CFU/g, with no contamination in both cultures (**Fig. 4.9A**). However, DASA03028 exhibited a viable cell count of $5x10^8$ CFU/g after 1 month, with contamination observed at $2x10^7$ CFU/g, while the viable cell count of strain SUTN9-2 was $1.2x10^9$ CFU/g, with contaminants present at $6x10^7$ CFU/g (**Fig. 4.9C**).

Remarkably, the FA technique, employing scFv antibodies, effectively determined the viable cell counts of individual *Bradyrhizobium* strain within mixed-culture peat-based inoculants. After combining these two strains at a 1:1 ratio, with an initial cell concentration of 10^8 CFU/g, the counts for DASA03028 and SUTN9-2 were $3.2x10^8$ and $4.4x10^8$ CFU/g, respectively at 0 month (**Fig. 4.9B)** while the counts for DASA03028 and SUTN9-2 remained at $6.5x10^5$ and $1x10^7$ CFU/g, respectively, following 1 month of storage, with contamination detected at $1.25x10^6$ CFU/g (**Fig. 4.9D**). These results indicate the advantages of using FA technique for quality control of inoculant with reduce the time of detection.

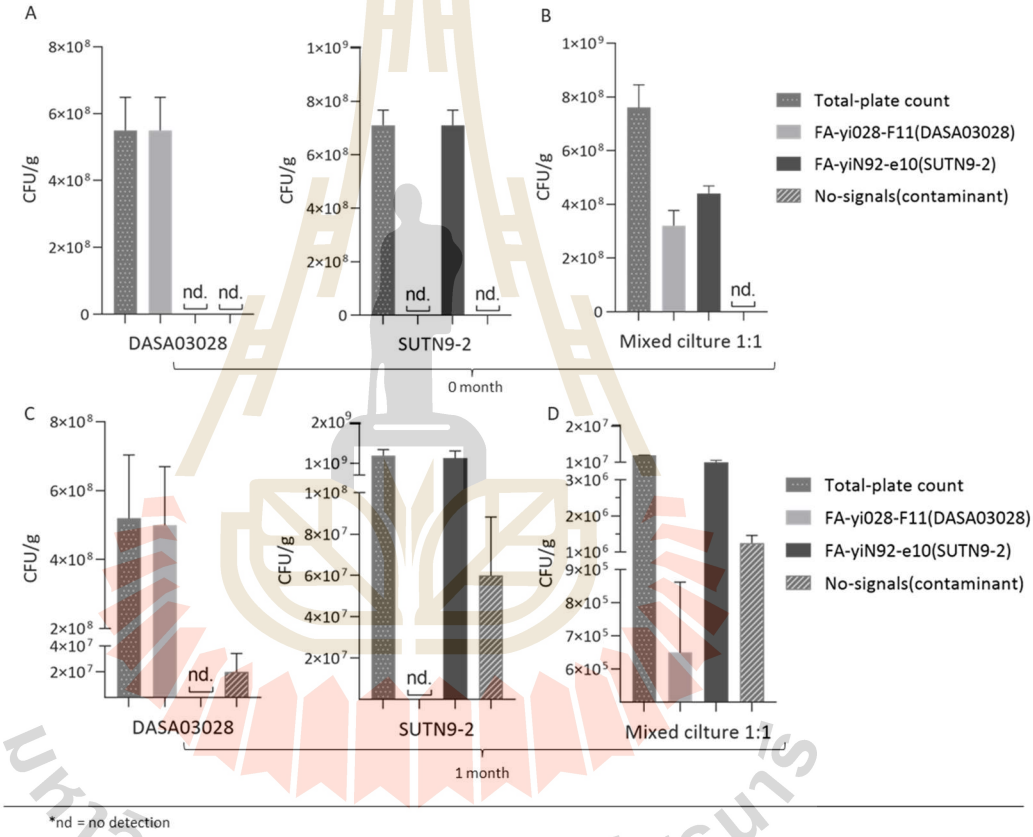

Figure 4.9 Determination the cell number of *Bradyrhizobium* sp. DASA03028 and SUTN9-2 in (A) single and (B) mixed cultures peat- base inoculant using FA technique after storage for 0 month, while (C) single and (D) mixed cultures peat-base inoculant using FA technique after storage for 1 month.

4.6 Detection of bradyrhizobium bacteroid in the nodule

The application of scFv antibodies for bradyrhizobium detection in the nodule was evaluated using ELISA technique. Surprisingly, the result indicated the cross-reactivity of antibody with some antigens presented in peanut nodule, but no cross-reactivity was detected in bacteroid from mung bean nodule. The bacteroid detections using scFv antibodies were also examined various varieties of peanut and mung bean (**Fig. 4.10**). The results confirmed the cross-reactivity of both scFv antibodies yi028-F11 and yiN92-e10 with some antigens presented in peanut nodules from all varieties (**Fig. 4.10A**). However, the detection of mung bean nodule bacteroid was successful in all varieties of mung bean tested in this study (**Fig. 4.10B**).

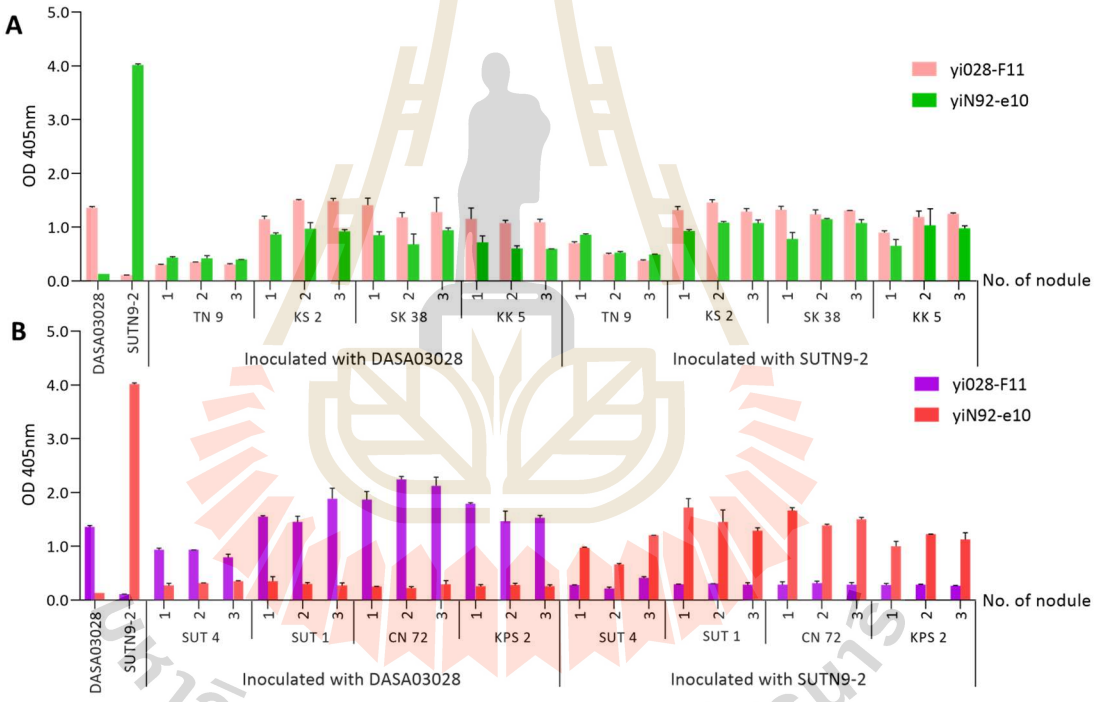

Figure 4.10 Evaluation of bacteroid detection in nodule of different legume varieties. scFv ELISA of yi028-F11 against *Bradyrhizobium* strain DASA03028 and yiN92-e10 against *Bradyrhizobium* SUTN9-2 were examined in nodules of (A) peanut varieties TN9, KS2, SK38, KK5; and (B) mung bean varieties SUT4, SUT1, CN72, KPS2. The detection was performed in each variety with 3 nodules to check the binding of scFv with bacteroid. Values are the mean of duplicate wells. Error bars show the standard deviation for each set of data.

The immunofluorescence assay was also used to confirm the cross-reactivity of peanut nodule (**Fig. 4.11A**). The positive signal was observed on peanut bacteroid regardless of

bradyrhizobial strains or scFv antibodies used. However, no cross-reactivity was detected in mung bean nodule bacteroid (**Fig. 4.11B**).

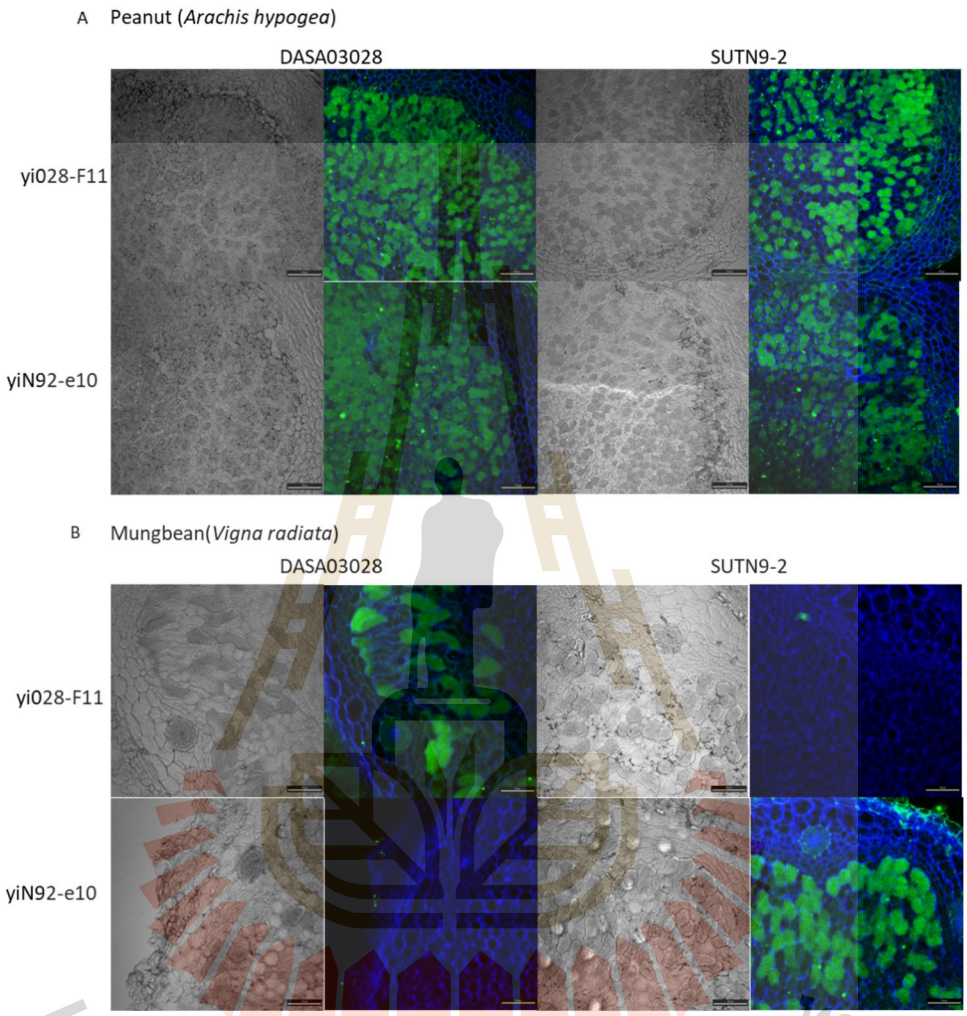

Figure 4.11 Confocal laser scanning micrographs of (A) peanut KS2 and (B) mung bean CN72 nodule inoculated with DASA03028 and SUTN9-2 staining with FA. The cross sections of nodule were stained with scFv yiN92-1e10 and yi028-F11. Green spot indicated the green-fluorescent staining of scFv antibody, using secondary antibody conjugated with anti-His Dylight 488. Plant cell walls were stained with a blue fluorophore (Calcofluor white M2R) and emitted blue color. The bacteroid cells are shown as green spots inside a blue plant cavity.

4.7 Determination of nodule occupancy by scFv antibody ELISA

In this study, the experiment was performed only in mung bean to evaluate the application of scFv antibodies for nodule occupancy determination using mixed culture

inoculant. The results were shown in **TABLE 4.2.** Bradyrhizobium sp. strains DASA03028 and SUTN9-2 have no significant ability in nodule occupancy when inoculated with mixed culture. This result indicated the possible application of scFv antibodies for further detection of nodulation competitiveness in the field practice.

Table 4.2 The analysis of mung bean nodule occupancy of DASA03028 and SUTN9-2

Plant inoculated with	**Nodule occupancy %**		
	DASA03028	SUTN9-2	Co-occupied
DASA03028	100±0	0±0	0±0
SUTN9-2	0±0	100±0	0±0
Mixed culture (1:1)	28.2±8.7	22.3±18.3	49.5±19.8

The values are the mean of 2 replications of ELISA with 4 plants.

CHAPTER V
DISCUSSION

5.1 Affinity selection (biopanning) and verification of phage displayed scFv antibodies against each strain of peanut *Bradyrhizobium* sp.

Naïve human library, the YAMO-I library was used in this study. This library was constructed from 140 non-immunized (naïve) donors (Pansri et al., 2009) for screening of specific antibody against to five peanut bradyrhizobia from DOA, resulting in the selection of a specific scFv antibody clone (yi028-F11) against strain DASA03028 after 4 rounds of bio-panning (3.69 fold when compared with control). This result was confirmed again about the possibility of isolation the antibody specific for bacterium in the genus *Bradyrhizobium* by screening from naïve human library, after it was ready to be used for screening of specific antibody for *Bradyrhizobium* sp. strains DOA9 and SUTN9-2 (Khaing et al., 2021; Vu et al., 2017). However, it was noticed that only one from five peanut bradyrhizobial strains was successfully isolated from naïve human library in this study **(Table 1).** This might indicate the presence of some specific epitope protein on cell surface of each bradyrhizobial strain (Bolaños et al., 2004). It is possible that these proteins were not related to human immune, while the surface proteins of some bradyrhizobia may similar with protein of human pathogen and resulting in successfully isolate scFv antibodies from the naïve human library. However, it is important to note that the ELISA signal of phage-displayed scFv clones yi028-F11 with *Bradyrhizobium* sp. strain DASA03028 was much lower than that of yiN92-e10 which specific to the strain SUTN9-2 **(Fig. 4.2).** This may be due to the differences in the affinity binding interaction between each antigen-antibody, or a high number of target protein that specific for yiN92-e10 was presented on bacterial cell membrane of strain SUTN9-2. However, this problem could be solved by increase the concentration of both target cell and antibody (Shah and Maghsoudlou, 2016), and the checkerboad titration was used to optimize the appropriate concentration of antigen and antibody (Min and Yamabhai, 2020). The ELISA signal of antibody clones yi028-F11 with strain DASA03028 could be increased upon the concentration of antibody and amount of antigen **(Fig. 4.4).** However, no cross-reactivity

of this scFv antibody clone with other bradyrhizobia or *Pseudomonas aeruginosa* **(Fig. 4.1B and Fig. 4.5)**, it indicates the potential of using this antibody in the filed application with no cross-reactivity with indigenous peanut bradyrhizobia.

5.2 Production of soluble scFv antibody

In this study the target antibody was produced in the *E. coli* SHuffle® T7. It should be noted that the level of expression was quite low and the expressed protein was found in both the soluble and inclusions bodies fractions **(Fig. 4.3).** Considerable efforts have been made in recent years to maximize the efficient production of soluble recombinant proteins in bacteria. The chemical complexity of the protein of interest can also play a role in its expression levels. Different proteins may have different optimal conditions for expression in *E. coli* (Ramkumar et al., 2017). The low level of protein expression may be due to the incompatible of codon usage in this strain of *E. coli* (Soleyman et al., 2016). However, the soluble protein was selected for purification to avoid denature and protein refolding. Thus, the suitable host cell should be considered for further production of antibody in the commercial scale.

5.3 Optimization of antibody-bacterial cell concentration and determination of cross-reactivity with other peanut *Bradyrhizobium* strains

The checkerboard titration was used to optimize the appropriate concentration of antigen and antibody (Min and Yamabhai, 2020). The ELISA signal of antibody clones yi028-F11 with strain DASA03028 could be increased upon increasing the concentration of antibody and amount of antigen (**Fig. 4.4**). At the high concentration, no cross-reactivity of this scFv antibody to *P. aeruginosa* or other bradyrhizobia was observed (**Fig. 4.1B and Fig. 4.5**), indicating that this scFv will be applicable for filed application where there will be no cross-reactivity with indigenous peanut bradyrhizobia.

5.4 The influence of bacterial cell aging on antibody binding ability

This result highlighted the relationship between the age of antigen cells and the binding property of recombinant single-chain variable fragment (scFv) antibodies. The study investigates this phenomenon using two different strains, SUTN9-2 and DASA03028, grown under solid medium (colony formed) and liquid culture, at different time points. For DASA03028, the findings indicate that the binding of the recombinant yi028-F11 scFv

antibody is influenced by the age of the cells and the cell forming. Interestingly, the antibody exhibited stronger binding with cells from liquid culture compared to those from colony-forming on solid medium. The optimal binding occurred at different time points: 7 days after inoculation for colony-form cells **(Fig. 4.6A)** and 30 days after inoculation for liquid culture cells **(Fig.4.6C)**. Despite the variation in binding efficiency, cells from colonies aged between 5 and 30 days remained detectable by ELISA. Conversely, the recombinant yiN92-e10 scFv antibody, specific to strain SUTN9-2, displayed better binding for colony culture **(Fig. 4.6B)** compared to liquid culture **(Fig.4.6D)**. Colony-form cells of different ages showed similar binding efficiency. Notably, cells from liquid culture of strain SUTN9-2 at 15 days after inoculation produced the highest signal, consistently more than two-fold higher than the non-target antigen, even when the cell age deviated from 15 days. So far, there have been report that several monoclonal antibodies reacting with core component lipopolysaccharide (LPS) from *Rhizobium leguminosarum* (Lucas et al., 1996; Wright, 1990). Typically, the surface of bradyrhizobia is known to contain various components, such as extracellular polysaccharides (EPS), capsular polysaccharides (CPS), K-antigens, flagella proteins, H-antigens, as well as lipopolysaccharides (LPS) or O-antigens (Carlson, 1984), while the K and H antigens are heat-labile (Somasegaran and Hoben, 2012). It is possible that the strain-specific cell surface molecule of strain DASA03028 that binding with antibody yi028-F11, may change their structure or chemical component along with cell growth. Moreover, the expression of a cell surface antigen in rhizobium is also regulated by oxygen and which may affect the antibody affinity (Kannenberg and Brewin, 1989). Thus, it is interesting to further discover the epitope on bradyrhizobial cell membrane that interact with this antibody to understand the factor of colony aging on its affinity binding.

5.5 Application the recombinant scFv antibodies for determination the viable cell counts of individual bradyrhizobium in the mixed-culture liquid- and peat-based inoculant through the implement of fluorescent antibody (FA) technique

The main challenges of using a single-culture inoculant in field application are the ability of competition with native rhizobium strains and its survivability in different environmental condition which affect the nodulation effectiveness (Zahran, 1999). To overcome these challenges, mixed cultures of rhizobium has been proved to increase the

efficiency of rhizobium inoculant. The utilization has been reported that co-inoculation of two symbiotically efficient *Bradyrhizobium* strains improves cowpea development better than a single bacterium application (do Nascimento et al., 2021). However, one challenge of using mixed culture inoculant is the quality and quantity of each strain mixed in the inoculants. Some strains may contain lower number of viable cell than others after storage. This may be cause from antagonistic properties of selected strain or the presence of contaminants (Rajasekhar et al., 2016). This can reduce the effectiveness of the inoculant and lead to poor efficiency. Typically, the traditional way to determine viable cell count is using plate count technique on selective medium, but it is difficult to distinguish individual *Bradyrhizobium* strains from a mixed-culture (Ph and Hoben, 1994). Although there are several methods for detection of individual strain, such as homology of amplified DNA, biochemical profile, protein profile, antibiotic profile, plasmid profile, and serological properties (Nick and Lindström, 1994), some of them are cost consume and not high throughput technique for strain monitoring. *Rhizobium* strain tagging with reporter genes, such as green fluorescent protein (GFP) is also used for monitoring rhizobium but somehow unacceptable to be used in general because of the GMO reason. Thus, the serology or immunological technique seems to be a simple and rapid when compared to microbiological or DNA-based techniques (Vu et al., 2017). However, the drawback of this technique is the non-distinguishable between viable and dead cells. The application of recombinant single-chain variable fragment (scFv) antibodies for determining the viable cell count of individual *Bradyrhizobium* strains in mixed-culture through the implementation of the fluorescent antibody (FA) technique is a new strategy for determination of both quality and quantity of rhizobial inoculant. This study demonstrated the utility of the FA technique in assessing the viable cell counts of *Bradyrhizobium* strains in both single-culture and mixed-culture inoculants **(Fig. 4.7).** It revealed in **Figure 4.8 and 4.9** that over the time, the viable cell counts of some strain increase over the other strain, which is crucial information for continue improving the effectiveness of mixed culture inoculants in agricultural applications. Additionally, the technique allowed for the early detection of potential contaminants, ensuring the quality of the inoculants. The results indicate that certain *Bradyrhizobium* strains remained viable and active even after extended storage periods, suggesting the stability and shelf-life of liquid inoculants. However, the compatibility of selected strains is very important in mixed culture inoculant. Here, it was found that the number of viable cell count of strain SUTN9-2 significantly

increased in the single inoculant, while this strain exhibited a decrease in cell count after 1 month in the mixed culture liquid inoculant with strain DASA03028 **(Fig. 4.8)**. This indicates potential interactions or competition between the strains. In case of peat-base inoculants which known to be susceptible to contamination, the compatibility of these two selected strains showed a decrease in cell counts for both strains compared to their single culture counterparts after 1 month, indicating possible competition or limited growth in this specific environment or contamination strain especially in case of DASA03028 **(Fig. 4.9).** However, the study successfully applied the FA technique to assess the quality of these inoculants by detecting the viable cell number of both *Bradyrhizobium* strains and contaminants.

5.6 Detection of of *Bradyrhizobium* bacteroid in the nodule

The utilization of scFv antibodies for monitoring bradyrhizobia after physiology change in nodule has been reported which successful in mung bean (*Vigna radiata*), *Aeschynomene americana* (Khaing et al, 2021) and siratro (*Macroptilium atropurpureum*) (Vu et al, 2017). However, this study it was found that detection of bacteroid after physiological changes in peanut (*Arachis hypogaea*) nodule was unsuccessful. The cross-reactivity of scFv antibodies with bacteroid antigens in peanut nodules was detected both with FA and ELISA techniques. So far there have been reported about monoclonal antibodies to antigens in the peribacteroid membrane from *Rhizobium*-induced root nodules of *Pisum sativum* cross-react with plant plasma membranes and Golgi bodies (Brewin et al., 1985). None of the existing research addresses how monoclonal

antibodies interact with the surface epitopes of rhizobia, especially after significant physiological changes when rhizobia are growing or inside nodules as bacteroid. In this study, the antibody could not be used to detect bacteroid in peanut nodules in all tested cultivars **(Fig. 4.10A)**. However, this phenomenon did not occur in mung bean nodules although different varieties were used **(Fig. 4.10B)** leading to similar results in the immunofluorescence assay **(Fig. 4.11)**. This result indicates the possibility of cross-reactivity between antibody with some molecules presented in peanut nodules. On the other hand, it has been known that there is the cell differentiation of peanut bradyrhizobial bacteroid into spherical shape (Chen et al., 2023). It is also possible that there are some changes on surface membrane of bacteroid and affect the binding of antibody. Future research efforts should focus on enhancing the antibodies specificity for nodule detection and exploring alternative detection methods.

5.7 Determination of nodule occupancy by scFv antibody ELISA

The utilization of recombinant human antibody to determine nodule occupancy are resulting in **Table 4.2.** The observed nodule occupancies indicate that there is a discernible competition occurring between these strains for symbiosis with the mung bean host. This competition may be driven by various factors, such as strain-specific interactions with the host plant, differences in nodulation efficiency, or variations in nitrogen fixation capabilities (Clúa et al., 2018). This understanding of competitive dynamics can guide the development of mixed or co-inoculant formulations that leverage the strengths of both strains to enhance overall symbiotic performance. By strategically combining strains with complementary traits, such as high nodulation efficiency and nitrogen fixation capacity, farmers can optimize nitrogen availability in their fields while minimizing the risk of nodulation failure or inefficient symbiosis.

CHAPTER VI

CONCLUSION

The development and application of scFv antibodies for the specific detection and quantification of peanut *Bradyrhizobium* strains represent a significant advancement in agricultural research and quality control of inoculant production. These antibodies offer high specificity and can be utilized in various applications, especially for detection of viable cell count. However, further improvements in specificity for nodule detection will contribute to their broader adoption in research and agricultural practices, ultimately benefiting legume crop production.

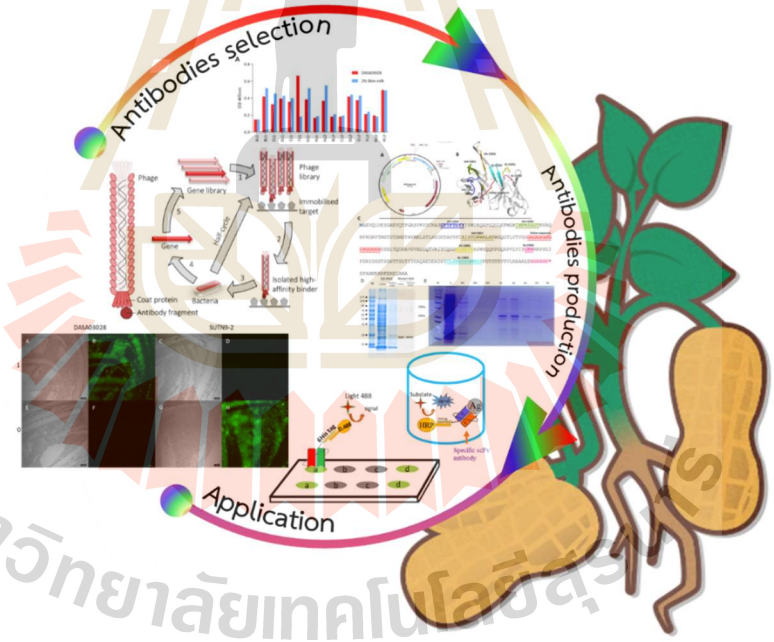

Figure 6.1 Overview of the development and application of scFv antibodies for advancement in agricultural research and quality control of peanut inoculant production.

APPENDIX

Table A1 List of materials for Luria Broth (LB) (Bertani, 1951)

Materials	Recipes
Tryptone	10 g
Yeast Extract	5 g
NaCl	10 g

Dissolve components in 1 liter of distilled or deionized water.

For LB agar* add agar to a final concentration of 1.5%.

Heat the mixture to boiling to dissolve agar and sterilize by autoclaving at 15 psi, from 121-124°C for 15 minutes.

Table A2 List of materials for YM (Somasegaran & Hoben, 2012)

Materials	**Recipes**
Mannitol	10 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
Yeast Extract	0.5 g
NaCl	0.1 g

Dissolve components in 1 liter of distilled or deionized water.

For YM agar* add agar to a final concentration of 1.5%.

Heat the mixture to boiling to dissolve agar and sterilize by autoclaving at 15 psi, from 121-124°C for 15 minutes.

Figure A1 Agarose gel electrophoresis of BOX-PCR fingerprinting patterns of 25 peanut *Bradyrhizobium* isolates

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