ESTABLISHMENT OF *Mucuna bracteata* AS A PLATFORM FOR THE PRODUCTION OF ANTI-TOXOPLASMA IMMUNOGLOBULIN

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Field of Study:

PLANT BIOTECHNOLOGY

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ESTABLISHMENT OF *Mucuna bracteata* AS A PLATFORM FOR THE PRODUCTION OF ANTI-TOXOPLASMA IMMUNOGLOBULIN

ABSTRACT

Production of recombinant proteins using plant system has gained a continuous momentum in the past decade. Traditionally, recombinant proteins are commercially produced through mammalian cell lines and microbial fermentation. These conventional platforms have intricate scalability, safety issues and costly. On the other hand, plants are easily scalable, economically competitive and do not harbor animal or human pathogens. As eukaryote, plants are capable to carry out complex post-translational modifications that are necessary for functional biological activities such as disulphide bridging and glycosylation. The aim of this study is to establish a new platform for the production of recombinant pharmaceutical protein in *Mucuna bracteata*. *M. bracteata* is an important perennial cover crop species that is widely planted as an underground cover in oil palm and rubber plantations. They have high biomass, grow vigorously and as a legume, able to fix nitrogen which are all ideal characteristics as a platform for mass production of recombinant protein. However, low germination rate and poor viability of *M. bracteata* seeds pose significant challenges of using the seeds as starting material for this study. To address these limitations, seed germination conditions (such as scarification period, surface sterilization protocols and imbibition period) were optimized and *in vitro* propagation protocols for *M. bracteata* was first established in this study. The results showed that seeds treated with sulphuric acid for 30 min, imbibed for 6 h and incubated in dark conditions on a wet cotton roll supplemented with 0.1% activated charcoal produced the highest percentage of seed germination (44%). *In vitro*-derived cotyledonary nodes showed the highest number of shoots per explant (5.60) and rooting response (92.9%) when cultured on Murashige and Skoog medium containing 4.44 μM 6-benzylaminopurine and 10.7 μM 1-naphthaleneacetic acid, respectively. The

optimized seed germination condition and *in vitro* protocol were used to develop stable and transient expression system of anti-toxoplasma immunoglobulin (IgG) in *M. bracteata*. In stable expression system, the highest percentage of transgenic shoots was generated when five day-old *M. bracteata* cotyledonary node explants were infected for 20 min and co-cultivated for 6-day with *Agrobacterium tumefaciens* strain GV3101. Unfortunately, the generated transgenic shoots have stunted growth that hindered extraction and quantification of anti-toxoplasma IgG. On the other hand, in transient expression system, vacuum infiltration of five week-old *M. bracteata* plant with *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 produced the highest concentration of anti-toxoplasma IgG in its bottom trifoliate leaf at two days post infiltration. When compared to the model plants for the production of plant-derived heterologous protein, *M. bracteata* consistently produced at least twofold higher concentration of anti-toxoplasma IgG than *Nicotiana benthamiana* and transgenic line of *Nicotiana tabacum* cv. SR1. Even though *M. bracteata* demonstrated the existence of structural heterogeneity in the purified anti-toxoplasma IgG, the anti-toxoplasma IgG retained its antigen binding properties as shown through ELISA. This study has demonstrated the feasibility of transient over stable expression system and laid the foundation towards establishing *M. bracteata* as a potential platform for the production of recombinant pharmaceutical protein via transient expression approach.

PENGGUNAAN *Mucuna bracteata* SEBAGAI PLATFORM UNTUK PENGHASILAN IMMUNOGLOBULIN ANTI-TOKSOPLASMA

ABSTRAK

Sejak dekad yang lalu, pengeluaran protein rekombinan menggunakan sistem tumbuhan telah mendapat momentum yang berterusan. Secara tradisinya, protein rekombinan dikeluarkan secara komersil melalui talian sel mamalia dan penapaian mikrob. Platform konvensional ini mempunyai isu keselamatan, skalabiliti yang rumit dan mahal. Sebaliknya, tumbuh-tumbuhan mudah dinaik skala, kompetitif dari segi ekonomi dan bukan hos semulajadi bagi patogen haiwan atau manusia. Sebagai eukariot, tumbuh-tumbuhan mampu memodifikasi protein selepas proses translasi secara kompleks seperti penyebaran disulfida dan glikosilasi yang penting untuk aktiviti biologi. Tujuan kajian ini adalah untuk mewujudkan satu platform baru bagi penghasilan protein rekombinan farmaseutikal menggunakan *Mucuna bracteata*. *M. bracteata* ialah spesies tumbuhan yang ditanam secara meluas sebagai tanaman tutup bumi di ladang kelapa sawit dan ladang getah. Mereka mempunyai jisim yang tinggi, kadar tumbesaran yang pantas dan sebagai legum, mampu mengikat nitrogen, yang mana merupakan ciri-ciri platform untuk pengeluaran protein rekombinan secara besar-besaran yang ideal. Walau bagaimanapun, kadar percambahan dan kebolehhidupan yang rendah dalam biji benih *M. bracteata* menimbulkan cabaran yang besar dalam penggunaan biji benih sebagai bahan permulaan untuk kajian ini. Untuk menangani batasan-batasan ini, faktor yang mempengaruhi percambahan biji benih (seperti tempoh sterifikasi, protokol pensterilan permukaan dan tempoh penyerapan) telah dioptimumkan dan protokol propagasi secara *in vitro* untuk *M. bracteata* telah dibangunkan pada peringkat awal kajian ini. Keputusan menunjukkan bahawa biji benih yang dirawat dengan asid sulfurik selama 30 minit, menjalani proses penyerapan selama 6 jam dan diinkubasi dalam keadaan gelap di atas kapas basah yang ditambah dengan 0.1% arang yang diaktifkan menghasilkan

peratusan percambahan biji benih (44%) yang tertinggi. Nod kotiledon yang diperolehi secara *in vitro* menghasilkan bilangan pucuk (5.60) dan respon perakaran (92.9%) yang tertinggi apabila dibiakkan menggunakan medium Murashige dan Skoog yang mengandungi 4.44 μM 6-benzylaminopurine dan 10.7 μM 1-naphthaleneacetic acid. Keadaan percambahan benih yang optimum dan protokol *in vitro* yang diwujudkan kemudiannya digunakan untuk membangunkan sistem ekspresi immunoglobulin anti-toksoplasma (IgG) dalam *M. bracteata* secara stabil dan sementara. Dalam sistem ekspresi stabil, peratusan tertinggi pucuk transgenik dihasilkan apabila nod kotiledon *M. bracteata* yang berusia lima hari dijangkiti selama 20 minit dan diinkubasi bersama selama 6 hari dengan *Agrobacterium tumefaciens* strain GV3101. Malangnya, pucuk transgenik yang dihasilkan mengalami perencatan pertumbuhan yang menghalang pengekstrakan dan kuantifikasi IgG anti-toksoplasma. Sementara itu, dalam sistem ekspresi sementara, penyusupan *A. tumefaciens* strain GV3101 yang mengandungi pTRAkcHcLcTg130 secara vakum ke dalam tumbuhan *M. bracteata* yang berusia lima minggu menghasilkan kepekatan anti-toxoplasma IgG yang paling tinggi di dalam daun trifoliat pada kedudukan terbawah pada dua hari selepas penyusupan. Apabila dibandingkan dengan tumbuhan model untuk penghasilan protein heterologus, *M. bracteata* secara konsisten menghasilkan kepekatan anti-toksoplasma IgG sekurang-kurangnya dua kali lebih tinggi berbanding *Nicotiana benthamiana* dan jaluran transgenik *Nicotiana tabacum* cv. SR1. Walaupun anti-toksoplasma IgG dari *M. bracteata* menunjukkan kepelbagaian struktur, ia masih mengekalkan keupayaan untuk mengikat antigen seperti ditunjukkan melalui ELISA. Kajian ini telah membuktikan keunggulan sistem ekspresi sementara berbanding sistem ekspresi stabil dan meletakkan asas ke arah menjadikan *M. bracteata* sebagai platform yang berpotensi tinggi bagi penghasilan protein rekombinan farmaseutikal melalui pendekatan sistem ekspresi sementara.

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^{o}C	:	degree Celsius
µg	:	microgram
µL	:	microliter
µM	:	micro molar
-ve	:	negative
+ve	:	positive
%	:	percent
®	:	registered trademark symbol
×g	:	relative centrifugal force
™	:	trademark symbol
A280 factor	:	Molecular weight $_{prot.}$ / Molar extinction coefficient ($M^{-1}*cm^{-1}$) $_{prot.}$
Abs.	:	absorbance
Abs. 280	:	absorbance at 280 nm wavelength
ANOVA	:	one-way analysis of variance
BAP	:	6-benzylaminopurine
bar	:	bialaphos resistance gene
bp	:	base pair
BSA	:	bovine serum albumin
CDC	:	Centers for Disease Control and Prevention
cGMP	:	current Good Manufacturing Practices
CHS	:	chalcone synthase 5'-untranslated region
cm	:	centimeter
C_{prot}	:	protein concentration (mg/mL)
CRISPR	:	Clustered Regularly Interspaced Short Palindromic Repeats

dH_2O	:	distilled water
DNA	:	deoxyribonucleic acid
ELISA	:	Enzyme-Linked Immunosorbent assay
F	:	forward primer
Fc	:	Fragment crystallizable region
FDA	:	Food and Drug Administration
g	:	gram
gfp	:	green fluorescent protein
h	:	hour
Hc	:	heavy chain
hEGF	:	human Epidermal Growth Factor
Hg	:	inch of mercury
HIV	:	Human Immunodeficiency Virus
hpt	:	hygromycin phosphotransferase gene
HPV	:	human papillomavirus
HRP	:	Horseradish peroxidase
IFN-a2b	:	Interferon alfa 2b
Inc.	:	Incorporated
KDEL	:	endoplasmic reticulum retention signal
kb	:	kilo base
kDa	:	kilo Dalton
kPa	:	kilo Pascal
L	:	liter
LB	:	left border
LB media	:	Luria Broth
Lc	:	light chain

LPH	:	codon-optimized murine signal peptide of mAB24
m	:	meter
M	:	molar
mg	:	milligram
min	:	min
mL	:	milliliter
mM	:	millimolar
mol	:	mole
MS	:	Murashige and Skoog
MW	:	molecular weight
NAA	:	1-naphthylacetic acid
ng	:	nanogram
nm	:	nanometer
NPT	:	Neglected Parasitic Infections
nptII	:	neomycin phosphotransferase gene
OD_{600}	:	optical density of a sample measured at a wavelength of 600 nm
p	:	probability value
P35SS	:	CaMV 35S promoter with duplicated transcriptional enhancer
pAnos'	:	nopaline synthase gene polyadenylation signal
pat	:	phosphinothricin acetyltransferase gene
PCR	:	polymerase chain reaction
PEG	:	polyethylene glycol
pg	:	picogram
PVP	:	polyvinylpyrrolidone
PVPP	:	polyvinylpolypyrrolidone
rpm	:	revolutions per minute

R	:	reverse primer
R^2	:	coefficient of determination
RB	:	right border
rfp	:	red fluorescent protein
RNA	:	ribonucleic acid
s	:	second
SAR	:	scaffold attachment region of the tobacco RB7 gene
sdH_2O	:	sterilized distilled water
SDS	:	sodium dodecyl sulfate
SDS-PAGE	:	SDS-polyacrylamide gel
SSR	:	simple sequence repeats
T-DNA	:	transferred DNA
TBE	:	Tris/Borate/EDTA
TBST	:	Tris-Buffered Saline, 0.1% Tween 20
Ti	:	tumor-inducing
TSP	:	total soluble protein
U	:	unit enzyme
UK	:	United Kingdom
USA	:	United States of America
V	:	voltan
v/v	:	volume per volume
vir	:	virulence gene
w/v	:	weight per volume
YEB	:	Yellow Extract Beef
yfp	:	yellow fluorescent protein

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CHAPTER 1: INTRODUCTION

1.1 Background

Plant molecular farming using genetically engineered plants has become a new strategy to mass-produce high-value recombinant pharmaceutical proteins, such as monoclonal antibodies, vaccines, and enzymes (Khatodia & Paul Khurana, 2018). Although most of the clinically available proteins and drugs are derived from mammalian cells, microbes, and yeast, the long period for scaling up and high costs of the existing production systems to meet the high demand for biopharmaceuticals are limiting factors for the manufacturing capacity of these conventional cell-based expression systems (Holtz et al., 2015; Nandi et al., 2016). Furthermore, the use of mammalian cells as a production platform also raises concerns about its safety. Microbial hosts offer a lower production cost than mammalian cells, but they may not be suitable for post-translational processing which affects the biological properties of the processed proteins (Egelkrout et al., 2012).

In comparison, plants are able to produce complex functional proteins with therapeutic activity, such as human serum proteins, growth regulators, antibodies, vaccines, and hormones due to their ability to perform post-translational modifications (Obembe et al., 2011). Moreover, plant-based systems offer simpler growth requirements with little to no risk of contamination with human pathogens compared to cultured mammalian cells (Ma & Wang, 2012). The selection of plant host is an important consideration because each species harbors unique biological characteristics that can influence expression level of recombinant protein and downstream processing (Jamal et al. 2009). High biomass yields, rapid scalability and non-food and feed plant are characteristics that are often regarded.

In this study, *Mucuna bracteata* DC. ex Kurz was selected as the platform for the production of mouse-human chimeric immunoglobulin (IgG) against *Toxoplasma gondii*. *M. bracteata* belongs to the family Fabaceae (Leguminoceae). It is the cover crop of choice for rubber and oil palm plantation in South India and Southeast Asia due to its vigorous growth, high biomass and ability to tolerate drought and shade conditions (Mathews, 1998; Mendham et al., 2004). Additionally, *M. bracteata* is a non-food and feed plant unable to produce seeds outside its native climatic region (Chiu 2007). These traits are significant for plant molecular farming to reduce the chances of food chain contamination.

Despite its potential, there are limited records of biotechnological approaches in the study of *M. bracteata*. Thus, an *in vitro* propagation protocol was initially developed to facilitate subsequent experiments in this study. Several studies have reported different expression levels of recombinant protein between transient and stable expression systems in plants (Garabagi et al., 2012; Melnik et al., 2018; Teh et al., 2014a). Hence, protocols for an *Agrobacterium*-mediated transformation of *M. bracteata* for both transient and stable expression systems were also established in this study. The antibody expression level in *M. bracteata* was then compared with *Nicotiana benthamiana* Domin and *Nicotiana tabacum* L. cv. SR1 as the model plants for transient and stable expression systems, respectively. Further comparison in terms of production time which could implicate upstream costs between the different expression platforms was briefly discussed (Figure 1.1).

1.2 Objectives

i. To develop an *in vitro* propagation protocol for *M. bracteata.*

ii. To establish a transient expression system expressing anti-toxoplasma IgG for *M. bracteata* using an *Agrobacterium*-mediated transformation method.

iii. To establish a stable expression system expressing anti-toxoplasma IgG for *M. bracteata* using an *Agrobacterium*-mediated transformation method.

iv. To evaluate, analyze and compare the production of anti-toxoplasma IgG in *M. bracteata* to *N. benthamiana* as a model plant system for transient expression.

v. To evaluate, analyze and compare the production of anti-toxoplasma IgG in *M. bracteata* to *N. tabacum* cv. SR1 as a model plant system for stable expression.

1.3 Hypothesis

Mucuna bracteata produces a higher concentration of anti-toxoplasma IgG than tobacco as a model plant in stable and transient expression system via *Agrobacterium*-mediated transformation.

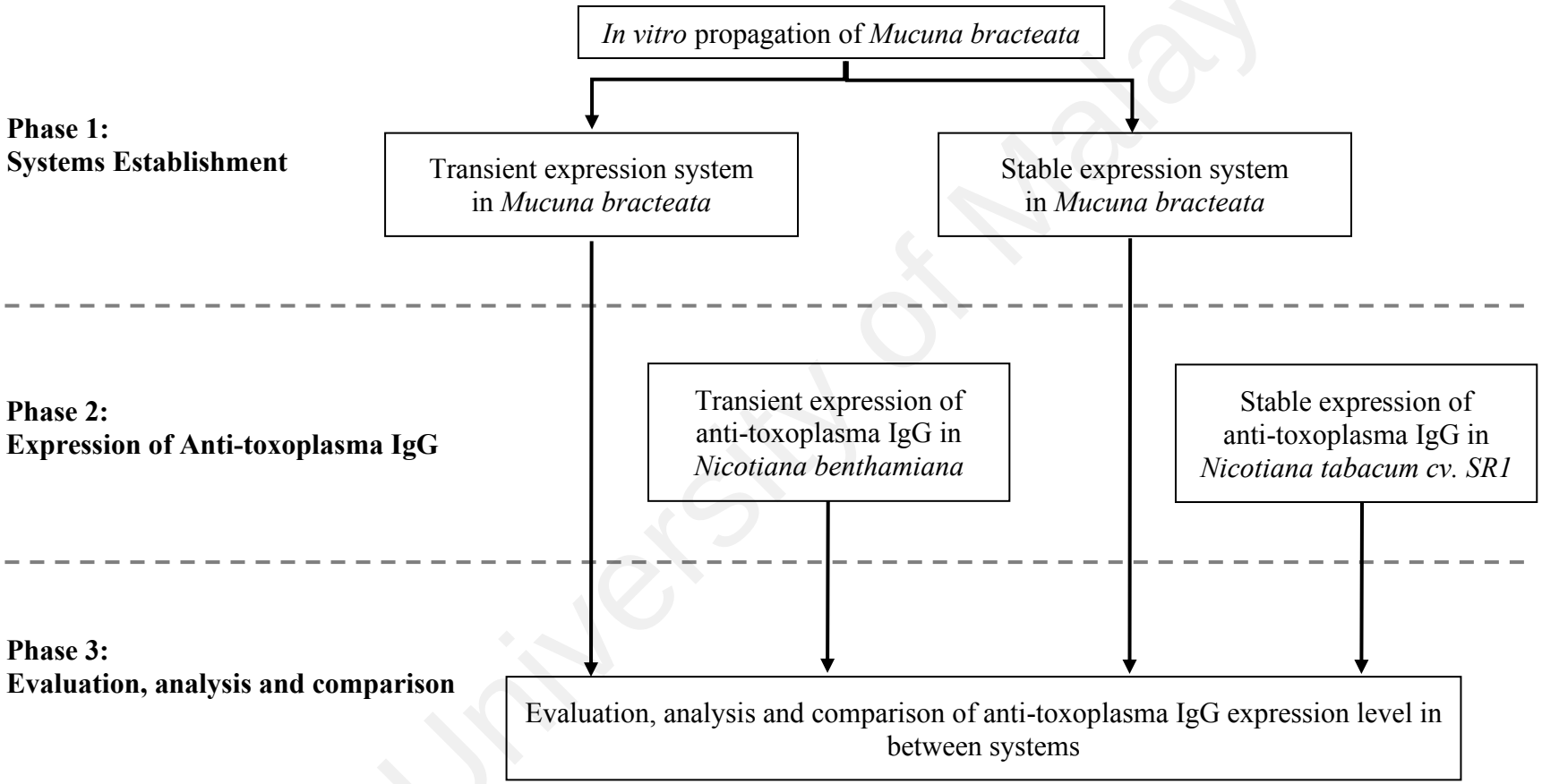

Figure 1.1 Overall flowchart of establishing *Mucuna bracteata* as a platform for the production of anti-toxoplasma immunoglobulin.

CHAPTER 2: LITERATURE REVIEW

2.1 Plants as a recombinant pharmaceutical protein production platform

Production of recombinant pharmaceutical proteins using plant systems also termed as molecular pharming or biopharming has gained continuous momentum in the past decade (Ma et al., 2015; Moon et al., 2019). Traditionally, recombinant pharmaceutical proteins are commercially produced through mammalian cell lines, yeast and bacterial expression system. These conventional platforms have several disadvantages such as requiring large capital, high maintenance cost, safety issues and intricate scalability (Melnik et al., 2018). In addition, recombinant proteins that are expressed in prokaryotic bacteria lack the complex post-translational modifications such as disulphide bridging and glycosylation that are necessary for functional biological activities (Nandi et al., 2016). Even though yeast is a lower eukaryote organism capable of conducting post-translational modifications, expression of full size IgG remains a challenge as it folds protein differently due to hyper-mannosylation during glycosylation (Çelik & Çalık, 2012; Frenzel et al., 2013). Furthermore, the most suitable yeast strain has to be identified and optimized at genetic and fermentative level for each individual recombinant pharmaceutical protein (Çelik & Çalık, 2012).

Plants, on the other hand, are cost-effective with low manufacturing costs and capital investment (Dirisala et al., 2016; Holtz et al., 2015; Nandi et al., 2016; Walwyn et al., 2015). Plants also do not harbor animal or human pathogens, thus eliminating the risks of product contamination with endotoxins (Xu et al., 2012; Yang et al., 2018). These biosafety advantages also impact some commercial aspects as they can reduce the purification costs and facility decontamination (Xu et al., 2012). Plants can be easily upscaled for mass production of crucial therapeutic proteins in times of bioterrorist attack or in an epidemic situation (Holtz et al., 2015). As a higher eukaryote organism, post-translational modifications in plants are largely similar to animals. Furthermore,

the process of producing recombinant proteins in plant system is convenient as the transgenic plants can be maintained at room temperature either in greenhouse or *in vitro* (Abiri et al., 2016; Moon et al., 2019). Among the least appreciated benefits of molecular pharming is the minimal technical expertise required during production, hence its transferability to developing countries and emerging economies to improve access to modern medicines (Ma et al., 2013; Ma et al., 2015). The advantages and disadvantages of various platforms for the production of recombinant pharmaceutical protein are summarized in Table 2.1.

Table 2.1: Comparison between various hosts in molecular pharming

	Plant cells	Transgenic plants	Animal cells	Transgenic animals	Yeast	Bacteria
Overall cost	Medium	Cheap	Expensive	Expensive	Medium	Cheap
Scale up capacity	Medium	High	Low	Low	High	High
Production cost	Medium	Low	High	High	Medium	Medium
Protein folding accuracy	High	High	High	High	Medium	Low
Product quality	High	High	High	High	Medium	Low
Protein yield	High	High	Medium	High	High	Medium
Safety	Medium	High	Medium	High	Unknown	Low
Public perception of risk	High	High	Medium	High	Medium	Low
Production vehicle	√	√	√	√	√	√
Multimeric protein	×	√	×	√	×	×
Glycosylation	Correct	Correct	Correct	Correct	Incorrect	Absent

*Modified from Abiri et al. (2016)

2.1.1 Plant species used for recombinant pharmaceutical proteins production

Various recombinant pharmaceutical proteins have been successfully expressed in many plant species. These include model plants such as *N. tabacum, N. benthamiana* and *Arabidopsis thaliana*; simple plants such as *Chlamydomonas reinhardtii* (algae) and *Lemna* (duckweed); leafy crops such as *Medicago sativa* (alfalfa), clover and *Lactuca sativa* (lettuce); cereals such as *Zea mays* (maize) and *Oryza sativa* (rice); legumes such as *Glycine max* (soybean), pea and pigeon pea; fruits and vegetables such as *Solanum tuberosum* (potato) and *Daucus carota* (carrot); and oil crops such as *Carthamus tinctorius* (safflower). Examples of recombinant pharmaceutical proteins that were expressed in these species are presented in Table 2.2.

In 2012, taliglucerase alfa that was produced in carrot suspension cultures for the treatment of Gaucher's disease becomes the first plant-derived pharmaceutical protein that was granted approval for human use by the Food and Drug Administration (FDA) (Fox, 2012; Tekoah et al., 2015). During the 2014-2016 outbreak of Ebola virus disease in West Africa, an experimental drug was administered to seven patients infected with Ebola. Five of them survived the infection. The experimental drug was a combination of three humanized monoclonal antibodies that recognized an Ebola virus surface glycoprotein and it was produced through transient expression in *Nicotiana benthamiana* plants (Qiu et al., 2014). Earlier in 2006, Cuban authorities have approved the use of tobacco-derived antibody as an affinity purification reagent for a hepatitis B vaccine produced in yeast (Melnik et al., 2018). Various others plant-derived pharmaceutical proteins are being developed with many currently at various stages of clinical development (Nandi et al., 2016).

Previously, there was a strong interest to express pharmaceutical proteins especially vaccines in edible plant organs such as potatoes and bananas as it provided significant advantages in term of scalability and logistical requirements compared to traditional vaccines production platforms (Kumar et al., 2005; Martín-Alonso et al., 2003; Youm et al., 2007). Plant-based edible vaccines provided a needleless, convenient and easy route of administration (Laere et al., 2016). Further advantages of molecular pharming could be observed from several reports of recombinant pharmaceutical protein production in cereal seeds. Recombinant proteins that were expressed in seeds were shown to remain stable and active for several years without significant degradation, eliminating the needs of expensive cold chain for storage and distribution network (Arcalis et al., 2014; Sabalza et al., 2013). However, heightened awareness of the risk for food supply contamination has led to a shift in preferences for non-food plants such as tobacco as plant bioreactors in recent years (Mazalovska et al., 2017; Menassa et al., 2012; Strasser et al., 2008; Sukenik et al., 2018; Wroblewski et al., 2005; Xiong et al., 2018).

2.1.2 Optimizing the yield of recombinant pharmaceutical protein in plant

Although plant-based systems have been used as an alternative platform for the production of recombinant pharmaceutical proteins, boosting protein yields remains a primary focus for economic feasibility (Abiri et al., 2016). Considering that heterologous protein expression level in genetically modified plant cells is a result of a series of complex biological events starting from the introduction of transgene primarily through *Agrobacterium*-mediated transformation to protein biosynthesis, accumulation in plant organs, harvesting and protein extraction and purification, there are incessant factors that could affect protein yield in biopharming (Buyel & Fischer, 2012; Fujiuchi et al., 2016; Habibi et al., 2017). As research in biopharming progresses, it is well established that the optimal conditions governed by these factors are highly dependent on the plant species and production strategy (Abiri et al., 2016; Melnik et al., 2018). In general, these factors can affect the protein yield either in the upstream or in the downstream segments of biopharming.

2.1.2.1 Factors affecting protein yield in the upstream segment

The upstream segment of biopharming generally involves designing the expression constructs, selection of plant host system, plant transformation and production strategy (either stable or transient systems) and determination of cultivation and harvesting parameters (Dirisala et al., 2016; Sack et al., 2015).

(a) ***Expression constructs***

Several factors are involved in controlling expression of a transgene at various molecular levels which includes transcription, translation, post-translational modifications or storage of recombinant protein in the cell (Sharma & Sharma, 2009). One of the key regulatory elements to achieve high level of transcription is the promoter. It contains the sequences which are required for RNA polymerase binding to drive transcription. The most commonly used promoter in plants transformation efforts is 35S from cauliflower mosaic virus which is of viral origin (Holden et al., 2010). It has long ago been reported by Kay et al. (1987) that doubling this sequence increased the transcription level approximately by tenfold. The transgene may also be fused to specialized transit peptide sequences to secrete the protein into a particular organelle such as endoplasmic reticulum and vacuole, oil bodies or even culture medium in plant roots (Cai et al., 2018; Chen et al., 2016; Madeira et al., 2016; Vafaee & Alizadeh, 2018). There are many other nucleic acid sequence elements that can also be added to a gene expression cassette to enhance expression such as translational enhancers, protease inhibitor and matrix attachment regions to increase stability and expression (Dietz-Pfeilstetter et al., 2016; Grosse-Holz et al., 2018; Rustgi et al., 2018; Thomas & Walmsley, 2014; Yamasaki et al., 2018).

(b) ***Plant host system***

Kang et al. (2017) has reported that recombinant protein yield varies not only between cultivars of the same species but also among *in vitro* regenerants derived from the same transgenic plants. This variation, coupled with consideration of the time needed for production, scalability and regulatory compliance of different platforms, has made it indispensable to select a favorable plant host system (Habibi et al., 2017). Selection of plant host system is usually based on certain criteria (Sharma & Sharma, 2009). Among the characteristics are the ability to produce high biomass yields, rapid scalability, non-food and non-feed crops, perennial plant, the ability to fix its own nitrogen, the ability to synthesize homogenous glycan structures, extended shelf life, easily contained, simple propagation and transformation procedures. However, it is not possible to obtain a perfect single system with all economic features as every system has its own advantages and disadvantages that have to be considered for optimal heterologous protein production (Frenzel et al., 2013; Habibi et al., 2017). Examples of plant species that have been used to express recombinant pharmaceutical proteins are listed in Table 2.2 and elaborated in Section 2.1.1.

(c) Plant transformation and production strategy

According to Tschofen et al. (2016), production of recombinant pharmaceutical protein in plants could be categorized into three major strategies (Figure 2.1):

i. Stable expression through cell suspension cultures
ii. Stable expression through transgenic lines
iii. Transient expression

In stable expression, transgene DNA that encodes for heterogeneous recombinant pharmaceutical proteins is integrated into the nuclear or plastid genome of plants tissue in a process known as transformation via either *Agrobacterium tumefaciens* (Section 2.2) or a physical delivery technique such as particle bombardment (Dirisala et al., 2016). Either way, the plants tissue could be cultured to establish plant cells suspension cultures or to generate whole transgenic plants. In whole transgenic plants, expression of recombinant pharmaceutical proteins could be localized to certain parts of the plants such as fruits, roots and leaves or even organelles such as endoplasmic reticulum through the usage of promoter (Dirisala et al., 2016; Song et al., 2018). *N. tabacum* is often used to establish stable transgenic lines expressing recombinant pharmaceutical proteins (Garabagi et al., 2012).

Between suspension cultures and transgenic plants, the latter is more scalable and cost-efficient (Tschofen et al., 2016). However, the process of generating both stable transgenic lines and suspension cultures is a time-consuming process (months to years) which is inappropriate for the required quick and large-scale production of vaccines against epidemics (Frenzel et al., 2013; Holtz et al., 2015; Nandi et al., 2016). Furthermore, stable transformation is a resource-intensive and inefficient process. Plants transformation often leads to heterogeneous cultures with inconsistent expression level of transgene protein, demonstrating the mixture of genetically and epigenetically

distinct cells within the regenerated plant cells (Kirchhoff et al., 2012; Philips et al., 2019). However, it was possible to screen regenerants from transformation events to select for elite transgenic lines or suspension cells with high expression level (Kirchhoff et al., 2012). Even so, prolonged cultivation, periodic subculturing of suspension cells, transgene silencing and recombination events may diminish or even abolish desirable traits such as the high expression level of recombinant pharmaceutical proteins (Dietz-Pfeilstetter, 2010; Philips et al., 2019).

In transient expression, transgene DNA that encodes for heterologous recombinant pharmaceutical proteins is not integrated into the plant's genome (Hwang et al., 2017). As long as the transgene DNA remains in the nucleus region, it can be transcribed to produce high level of desired proteins within a short period of time without the needs to regenerate and screen for transgenic plants which is often a time consuming process (Liu et al., 2019). There are several transient expression methods namely agroinfiltration, mesophyll-protoplasts PEG-mediated, plant virus infection or electroporation transfection (Kopertekh & Schiemann, 2019). The widely used method for transient expression is agroinfiltration in which *A. tumefaciens* is injected or vacuum infiltrated into the intercellular spaces of leaves (Habibi et al., 2017; Tschofen et al., 2016). Many plant species can be processed using this method, but the most common ones are *N. benthamiana* (Garabagi et al., 2012). Due to its versatility and capability to produce high yield of recombinant pharmaceutical proteins in a relatively short period of time (days), agroinfiltration has become the preferred transient expression platform to manufacture recombinant pharmaceutical proteins in plants (Norkunas et al., 2018). Several facilities for large-scale transient production based on agroinfiltration method have already been built in compliance with the current Good Manufacturing Practices (cGMP) and are currently operational (Holtz et al., 2015; Nandi et al., 2016).

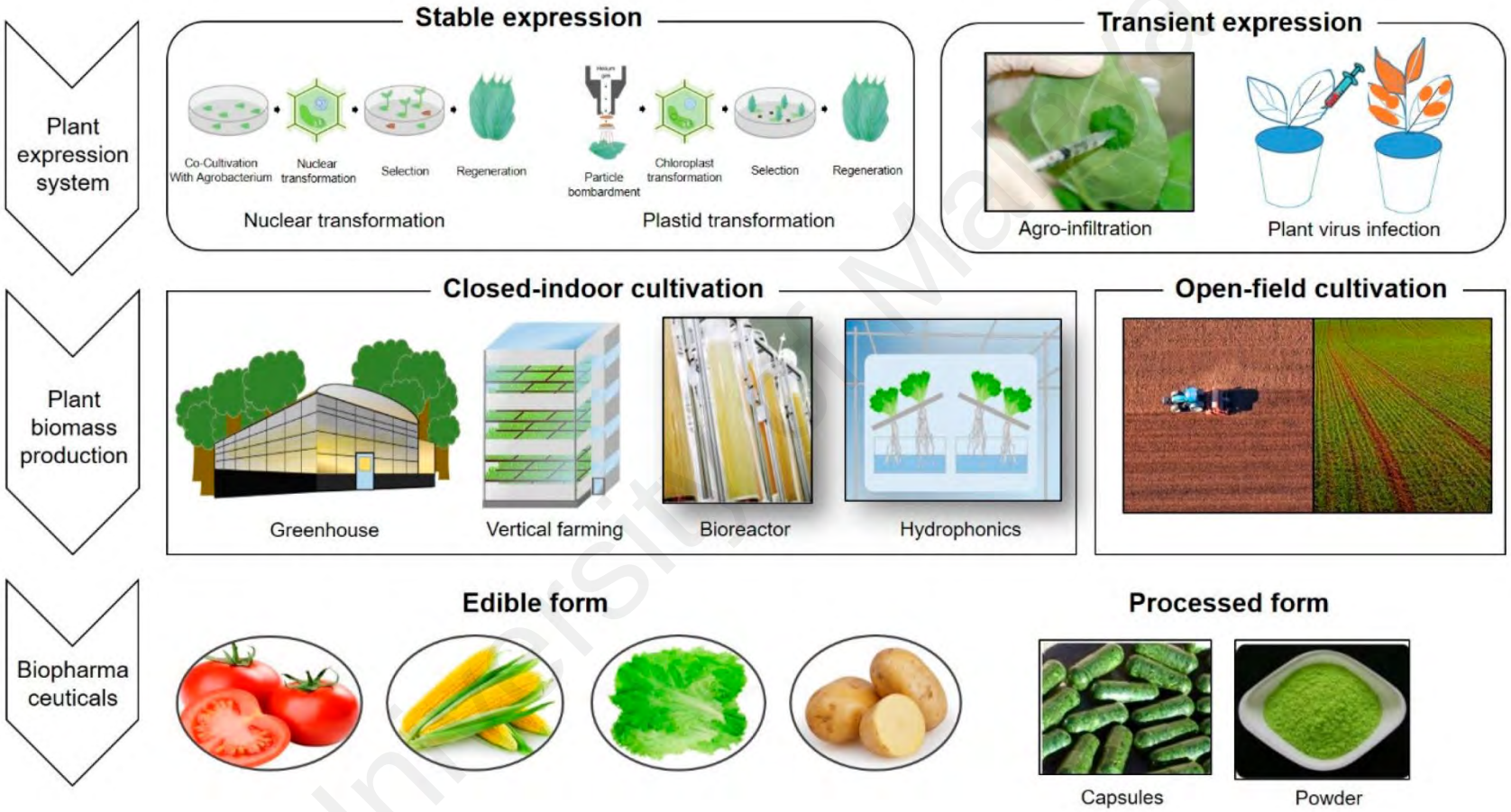

Figure 2.1: Overview of production system of plant-based recombinant pharmaceutical proteins. From Moon et al. (2019). Copyright by creative commons – Attribution 4.0 International (CC BY).

Nevertheless, as highlighted by Garabagi et al. (2012), every strategy adopted for the production of recombinant pharmaceutical protein in plants has its own strengths and weakness. Adoption of a particular strategy has to be suited for certain classes of recombinant proteins based on various factors including scale, regulatory compliance, cost-efficiency and upstream and downstream processing constraints of the targeted recombinant proteins. Indeed, another advantage of plant based production of pharmaceutical proteins compared to other conventional production platform is the diversity of the system which comprises of wide arrays of plant species that can be cultured in closed-indoor or open-field system and expressed either stably or transiently (Figure 2.1) (Moon et al., 2019). Such diversity makes it possible to respond rapidly to market needs by expressing novel recombinant proteins that could be prescribed in either edible or processed form (Figure 2.1) (Moon et al., 2019).

Table 2.2: Selected examples of pharmaceutical proteins expressed in plants

Pharmaceutical protein	Host plant	Application	Production strategy	References
Anti-toxoplasma immunoglobulin	*Nicotiana benthamiana*	Treatment of toxoplasmosis	Transient	Lim et al. (2018)
Apolipoprotein A-I	*Carthamus tinctorius* (safflower)	Treatment of vascular diseases	Stable	Nykiforuk et al. (2011)
	Nicotiana tabacum		Stable	Chiaiese et al. (2011)
Beak and feather disease virus coat protein	*Nicotiana benthamiana*	Therapeutic vaccines against BFDV	Transient	Regnard et al. (2017)
Bevacizumab monoclonal antibody	*Oryza sativa* (rice)	Treatment of multiple cancers by targeting vascular endothelial growth factor	Transient	Chen et al. (2016)
Cholera toxin B antigen	*Lactuca sativa* L. (lettuce)	Therapeutic vaccines against porcine epidemic diarrhea	Stable	Huy et al. (2011)c
Dengue virus NS1 antigen	*Nicotiana benthamiana*	Therapeutic vaccines against dengue	Transient	Marques et al. (2020)
Dengue virus serotypes 1-4 envelope glycoprotein	*Nicotiana benthamiana*	Therapeutic vaccines against dengue	Transient	Huy and Kim (2017)
Erythropoietin	*Nicotiana benthamiana*	Treatment of anemia, rheumatoid arthritis and other types of anemia	Stable	Jez et al. (2013)
	Nicotiana tabacum		Stable	Kittur et al. (2012)
Fibroblast growth factor 9	*Carthamus tinctorius* (safflower)	Hair growth, tissue repair, and burn wounds.	Stable	Cai et al. (2018)
Fibroblast growth factor 10			Stable	Huang et al. (2017)
Gastric lipase	*Arabidopsis thaliana*	Treatment of cystic fibrosis, pancreatitis	Stable	Guerineau et al. (2020)
	Brassica rapa (turnip)		Stable	Ele Ekouna et al. (2017)
Griffithsin	*Lactuca sativa* cv. TN-96-39 (lettuce)	Potent viral entry inhibitor particularly HIV	Stable	Vafaee and Alizadeh (2018)
	Nicotiana tabacum cv. Samsun		Stable	
Heat-labile enterotoxin B subunit and cholera toxin B subunit	*Oryza sativa* (rice)	Prevention of diarrhea	Stable	Soh et al. (2015)

Table 2.2 continued: Selected examples of pharmaceutical proteins expressed in plants

Pharmaceutical protein	Host plant	Application	Production strategy	References
Hepatitis B antigen	*Zea mays* (Maize)	Therapeutic vaccines against Hepatitis B	Stable	Hayden et al. (2012)
HEV genotype 3 ORF2 capsid protein	*Nicotiana benthamiana*	Treatment of Hepatitis E	Transient	Mazalovska et al. (2017)
Human butyrylcholinesterase	*Nicotiana benthamiana*	Treatment of organophosphate nerve agents such as sarin	Transient	Alkanaimsh et al. (2016)
Human epidermal growth factor	*Nicotiana benthamiana*	Treatment of chronic diabetes ulcers	Transient	Thomas and Walmsley (2014)
Human extracellular superoxide dismutase	*Nicotiana benthamiana*	Treatment of skin disease and arthritis	Transient	Park et al. (2016)
Human lactoferrin	*Medicago sativa* L. (alfalfa)	Iron absorption, natural protection against pathogens, anti-inflammatory activities, and modulation of the immune system	Stable	Stefanova et al. (2013)
Human Papillomavirus (HPV) 16 E7 antigen	*Chlamydomonas reinhardtii* (algae)	Therapeutic vaccines against HPV-related lesions and cancers	Suspension cells	Demurtas et al. (2013)
Human serum albumin	*Solanum tuberosum* cv. Désiree (potato)	Replace blood volume loss resulting from trauma	Stable	Farran et al. (2002); Sijmons et al. (1990)
Insulin-like growth factor	*Arabidopsis thaliana*	Neuropathy, aging and cancer treatment	Stable	Li et al. (2011)
Interferon alfa 2b (IFN-a2b)	*Daucus carota* (carrot)	Treatment of Hepatitis C, Herpetic infection	Suspension cells	Rosales-Mendoza and Tello-Olea (2015)
Interleukin-6	*Nicotiana benthamiana*	Regulation of immune responses, inflammation and hematopoiesis	Stable	Nausch et al. (2012)

Table 2.2 continued: Selected examples of pharmaceutical proteins expressed in plants

Pharmaceutical protein	Host plant	Application	Production strategy	References
Interleukin-10 (IL-10)	*Nicotiana tabacum*	Immunoregulation and inflammation	Stable	Sadeghi et al. (2016)
	Oryza sativa (rice)		Stable	Takagi et al. (2015)
M2e peptide of avian influenza virus H5N1	*Lemna minor* (duckweed)	Therapeutic vaccines against influenza A strains	Stable	Firsov et al. (2015)
Interleukin-6	*Nicotiana benthamiana*	Regulation of immune responses, inflammation and hematopoiesis	Stable	Nausch et al. (2012)
Interleukin-10 (IL-10)	*Nicotiana tabacum*	Immunoregulation and inflammation	Stable	Sadeghi et al. (2016)
	Oryza sativa (rice)		Stable	Takagi et al. (2015)
M2e peptide of avian influenza virus H5N1	*Lemna minor* (duckweed)	Therapeutic vaccines against influenza A strains	Stable	Firsov et al. (2015)
Monoclonal antibody 2F5	*Zea mays* (maize)	Neutralizing HIV	Stable	Sabalza et al. (2012)
Monoclonal antibody CMG2	*Nicotiana benthamiana*	Anthrax decoy protein	Transient	Xiong et al. (2018)
Monoclonal antibody CO17-1A	*Arabidopsis thaliana*	Treatment of colorectal cancer	Stable	Song et al. (2018)
Monoclonal antibody M12	*Nicotiana tabacum*	Marker for breast adenocarcinoma	Suspension cells	Kirchhoff et al. (2012)
Pf antigen	*Nicotiana benthamiana*	Malaria vaccine	Transient	Spiegel et al. (2015)
Staphylococcal endotoxin B	*Raphanw sativus* L.	Therapeutic vaccines against Staphylococcal	Transient	Liu et al. (2018)
Taenia solium HP6/TSOL18 antigen	*Daucus carota* (carrot)	Therapeutic oral vaccine against porcine cysticercosis	Stable	Monreal-Escalante et al. (2016)
Taliglucerase alfa	*Daucus carota* (carrot)	Treatment of Gaucher's disease	Suspension cells	Fox (2012); Tekoah et al. (2015)
Toxoplasma gondii GRA4 antigen	*Nicotiana tabacum*	Vaccine candidate for toxoplasmosis	Stable	del L. Yácono et al. (2012)
Toxoplasma gondii SAG1 antigen	*Nicotiana tabacum*	Vaccine candidate for toxoplasmosis	Stable	Albarracín et al. (2015)

(d) Cultivation and harvesting parameters

As reviewed by Moon et al. (2019), there are two different approaches to cultivate the transgenic plants expressing various recombinant pharmaceutical proteins: open-field cultivation and closed culture systems. In the open-field system, transgenic plants with stable expression of recombinant pharmaceutical proteins are cultivated in an open field without the need of expensive infrastructure. There is a huge potential risk of cross-pollination associated with this cultivation strategy as proven by an incident in Iowa, United State where 155 acres of corn have to be destroyed because it could have been cross-pollinated by ProdiGene's transgenic corn expressing recombinant pharmaceutical proteins being raised in a nearby field (Fox, 2003). In another case involving the same company, 500,000 bushels of harvested soybeans in Nebraska, United States were contaminated with small amount of transgenic maize seeds expressing avidin which had been grown on the same plot in the previous planting season (Fox, 2003). These incidents have not only led to a stronger and stringent regulation to govern cultivation of transgenic plants in an open-field but also a shift of preferences for non-food crops as the production platform in a closed culture systems (Fox, 2003; Moon et al., 2019).

In recent years, most transgenic plants expressing recombinant pharmaceutical proteins are cultivated in closed systems as a containment measure to prevent escape of transgenes. Greenhouses are one of the most promising systems for large-scale production of recombinant pharmaceutical protein in plants as they provide a compromise between capital investment and ease of scale-up (Moon et al., 2019). Several cGMP-certified facilities for large-scale production of plant-derived recombinant pharmaceutical proteins such as those of iBio CMO (formerly Caliber Biotherapeutics, Texas, USA), Franhoufer IME (Aachen, German), Protalix BioTherapeutics (Karmiel, Israel), Kentucky BioProcessing (Kentucky, USA) and

Medicago Inc. (North Carolina, USA) have already been built and are currently operational (Holtz et al., 2015; Nandi et al., 2016). Growing conditions in greenhouses such as light intensity and wavelength, temperature, planting density, humidity and nutrient supply can be controlled to optimize plant growth, development and accumulation of recombinant pharmaceutical protein (Buyel & Fischer, 2012; Fujiuchi et al., 2016). Furthermore, controlled environment allows for year-round cropping to maximize annual production output (Buyel & Fischer, 2012).

Another example of closed system is bioreactor culture system (Moon et al., 2019). Plant cells or organs such as roots are cultured in liquid media under sterile and controlled conditions. A huge advantage of bioreactor culture system is the ability to secrete target materials into the culture medium, thereby simplifying extraction and purification procedure to reduce costs (Madeira et al., 2016). Among the examples of recombinant pharmaceutical proteins that have been successfully produced using this system are IFN-a2b for the treatment of Hepatitis C in carrot (Rosales-Mendoza & Tello-Olea, 2015) and therapeutic vaccines against HPV-related lesions and cancers in algae (Demurtas et al., 2013). Important factors that have to be optimized include selection of the host plant, the type of plant material (suspension cells or hairy root), media components and the bioreactor type and operating method (Abiri et al., 2016; Moon et al., 2019). Similar to mammalian cell culture system, cultivation of plant cells or organs through bioreactor system requires a higher level of educated and experienced personnel for operation, sterilization, troubleshooting, and maintenance (Nandi et al., 2016).

Following cultivation, plant materials are harvested under optimal conditions to maximize heterologous protein yield. It has been well documented that expression levels of heterologous protein varies according to the plants age and accumulate differently in various parts of the plants (Sack et al., 2015). For instance, Thomas and Walmsley (2014) has reported that using 5-week-old *N. benthamiana* plants to transiently expressed human epidermal growth factor (hEGF) significantly increased expression compared to 4- or 6-week-old-plants. In addition, Sack et al. (2015) and Buyel and Fischer (2012) have reported that heterologous protein expression was highest in leaves at the intermediate position along the plant stem compared to leaves at other position. Similar observations where heterologous protein yield varies according to the plants' age and parts have commonly been reported (Buyel & Fischer, 2012; Jansing & Buyel, 2019; Jin et al., 2015; Yasmin & Debener, 2010).

2.1.2.2 Factors affecting protein yield in the downstream segment

In the downstream process of biopharming, recombinant pharmaceutical proteins are recovered from plant biomass through several processes of extraction, clarification and protein purification (Sack et al., 2015). Considering that the recombinant protein has to be exceptionally pure and conform to cGMP, downstream processing is the critical component in biopharming where it is estimated to account for up to 80% of the overall cost in biopharming (Łojewska et al., 2016; Wilken & Nikolov, 2012).

The extraction method depends largely on the type of plant host system and the production strategy employed (Łojewska et al., 2016). Wherein plant cell suspension cultures, aquatic plants and hydroponic whole-plant systems can secrete the recombinant proteins into the culture medium, recombinant proteins in majority of biopharming efforts are retained within the plant tissue which must be released into a buffer by tissue maceration (Tschofen et al., 2016). Additives such as protease inhibitors or PVP are often added into the buffer to minimize protein degradation and conformational changes due to proteases and phenolic compounds secretion during tissue maceration process (Charmont et al., 2005; Wang et al., 2007; Yao et al., 2006). The success of extraction process is governed by various extraction parameters (i.e. types of samples, choice of extraction techniques, solvent nature, buffer ionic strength and pH, presence of denaturing agents and temperature) that are correlated not only to the type of plant host system and the production strategy employed but also to the recombinant protein of interest which highlights the needs of stepwise optimization (Alkanaimsh et al., 2016; Menzel et al., 2016; Temporini et al., 2020).

Tissue disruption introduces numerous soluble and insoluble contaminants such as DNA, RNA, alkaloids, polysaccharides, polyphenols, chlorophyll and soluble proteins that must be removed or lowered to acceptable levels during clarification prior to purification of recombinant pharmaceutical protein (Sharma & Sharma, 2009; Tschofen et al., 2016). Among clarification techniques that are often used to remove these contaminants are centrifugation, filtration and precipitation techniques (Wilken & Nikolov, 2012). Regardless of the clarification techniques chosen, the protocols have to be carefully optimized, particularly for unstable proteins that are prone to degradation via oxidation, deamidation or proteolysis (Temporini et al., 2020).

Once the extract is clarified, recombinant pharmaceutical protein is purified via chromatography. There are many chromatography techniques in use currently, namely Protein A affinity chromatography, ion-exchange chromatography, hydrophobic interaction chromatography, hydrophobic charge induction chromatography or mixed mode chromatography (Temporini et al., 2020). In affinity chromatography, the recombinant protein forms a reversible interaction with a specific and stable ligand bound to a matrix support. The most commonly used ligand for the purification of monoclonal antibody is Protein A, a naturally occurring cell surface protein found in *Staphylococcus aureus* strains with high affinity towards the Fc region of an immunoglobulin (Arora et al., 2017). Due to its relatively simple and well-standardized procedures, Protein A affinity chromatography is the preferred method for monoclonal antibody purification either in small-scale or large-scale production of biopharming (Buyel et al., 2017; Hnasko & McGarvey, 2015; Sack et al., 2015; Temporini et al., 2020).

2.2 *Agrobacterium*-mediated transformation

A. tumefaciens is a Gram-negative bacteria that was first isolated from the gall tissue and identified as the cause of crown gall disease in plants (Smith & Townsend, 1907). *Agrobacterium* has a natural ability to transfer DNA fragment (transferred DNA or T-DNA) into plants which has been used as a tool to transfer favorable traits to crop plants to enhance quantitative and qualitative characteristics of agricultural production (Ziemienowicz, 2014). Ever since the first report on successful generation of transgenic plant using *Agrobacterium*-mediated gene transfer technique, a large number of transgenic plants with many new traits (such as resistance to insect or disease, herbicide resistance, biotic and abiotic stress tolerance, metal tolerance, high growth rate, higher yield, and higher nutritional quality) have been frequently reported (Guo et al., 2019; Zambryski et al., 1983). In the last two decades, *Agrobacterium*-mediated transformation has been widely adopted by biotechnological industry to express various heterologous recombinant proteins such as pharmaceutical proteins and industrial enzymes in plants (Fujiuchi et al., 2016; Holtz et al., 2015; Nandi et al., 2016).

Indeed, there are many other gene transfer technologies such as particle bombardment, electroporation, microinjection and chemical transduction but *Agrobacterium*-mediated transformation remains as the preferred method in plants genetic modification due to its versatility, low cost and higher efficiency (Bleotu et al., 2018). Rather than becoming obsolete, *Agrobacterium*-mediated gene transfer technique has become ever prominent as the mediator to deliver the advent CRISPR/Cas9 binary vector into plant cells for precise genome editing technology (Zhang et al., 2018; Zhang et al., 2019).

2.2.1 Mechanism of action

The *Agrobacterium*-plant interaction is a complex process that can be delineated into five key steps (Ziemienowicz, 2014):

i. Induction of the bacterial virulence system
ii. Generation of T-DNA complex
iii. Transfer of T-DNA from *Agrobacterium* to the host cell nucleus
iv. Integration of T-DNA into the plant cell genome
v. Expression of T-DNA gene(s) by the host cell

This entire process requires the presence of two genetic components; the T-DNA and the virulence (*vir*) region (Hwang et al., 2017). The T-DNA is flanked by two 25-base pair (bp) imperfect direct repeats called as left border (LB) and right border (RB) sequences. The *vir* region is composed of at least seven major loci (*virA, virB, virC, virD, virE, virF*, and *virG*) that encodes components for the gene transfer mechanism (Gordon & Christie, 2014). As the key player in the virulence induction, the VirA and VirG proteins activate the expression of other *vir* genes to process, transfer and integrate the T-DNA into the plant cell (Guo et al., 2019).

In naturally occurring systems, the T-DNA and the *vir* region are located on a tumor-inducing (Ti) plasmid of the *Agrobacterium*. Through genetic modification, a binary vector system composed of a small binary plasmid (containing the T-DNA) and a helper plasmid (containing the *vir* region) was developed to allow easier *in vitro* manipulation of the T-DNA region (Gordon & Christie, 2014). Besides the gene(s) of interest, the T-DNA region in the binary plasmid is often engineered to contain a selectable marker for the selection of transgenic plant (Hwang et al., 2017).

2.2.2 Factors affecting *Agrobacterium*-mediated transformation

Under natural conditions, *Agrobacterium* was observed to infect almost exclusively only some dicotyledonous plants. However, extensive studies on factors affecting *Agrobacterium*-mediated transformations and optimizing these transformation conditions have broaden the host range and increase the transformation efficiency in *Agrobacterium* natural hosts (Guo et al., 2019). Many plant species previously thought to be very resistant to *Agrobacterium*-mediated transformation such as monocotyledonous plants and legumes are routinely transformed today using *Agrobacterium*-mediated gene transfer technique (Atif et al., 2013; Hayta et al., 2019; Kimura & Isobe, 2018; Zhang et al., 2013).

It is well established that many protocols in plants research are not only species but also genotype dependent (Atif et al., 2013; Guo et al., 2019; Hisano et al., 2017; Wroblewski et al., 2005; Yasmin & Debener, 2010). Therefore, an optimal condition for *Agrobacterium*-mediated transformation must always be determined for novel plant material (Abiri et al., 2016).There are various factors that affect the efficiency of T-DNA transfer and its integration into the plant genome including genotype of the plant, type of explant, plasmid vector, bacterial strain and density, period and temperature of co-cultivation, composition of culture medium and usage of additives or surfactants (Abiri et al., 2016; Ziemienowicz, 2014). These factors are summarized in Table 2.3.

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Table 2.3: Factors influencing *Agrobacterium*-mediated transformation efficiency

Factors	Examples	References
Explant type	Root, shoot, cotyledon, embryo, hypocotyls, cell suspension	Alok et al. (2016); An et al. (2014); Sack et al. (2015); Yan et al. (2019)
Vector plasmid	pCAMBIA, pGreen, pGA, pCG, pGPTV, Bi-BAC, pTRA	Bashandy et al. (2015); Wu et al. (2014)
Bacterial strain	GV3101, LBA4404, EHA101, EHA105, C58, AGL1	Deeba et al. (2014); Liu et al. (2019); Liu et al. (2020); Sun et al. (2020)
Composition of culture medium	Salt concentration, sugars, growth regulators	Wu et al. (2014); Yan et al. (2019)
Temperature of co-cultivation	General range: 19 – 30 ^{o}C	Buyel and Fischer (2012); Liu et al. (2020); Zhang et al. (2013)
Period of co-cultivation	1-5 days Common: 24 h, 48 h, 60 h, 72 h	Buyel and Fischer (2012); Liu et al. (2020); Mortensen et al. (2019); Sun et al. (2020)
Agrobacterium density	$1 \times 10^{6} – 1 \times 10^{10}$ cfu/ml	Alok et al. (2016); An et al. (2014); Buyel and Fischer (2012); Liu et al. (2020)
pH of co-cultivation medium	Acidic pH: 5.2, 5.5, 5.6, 5.8 or 6.0	Wu et al. (2014)
Types of antibiotics and its concentrations to suppress *Agrobacterium* overgrowth	Cefotaxime, carbenicillin, timentin	Kumar et al. (2017)
Types of chemicals additives and its concentrations	Acetosyringone, L-cysteine, dithiothreitol, sodium thiosulphate, lipoic acid, ascorbic acid and PVP	Alok et al. (2016); An et al. (2014); Yang et al. (2016)
Types of surfactants and its concentrations	Silwet L77, pluronic acid F68, Tween20	Liu et al. (2019)
Selectable markers (*in the case of antibiotic and herbicide resistances, different plants would have different natural tolerance as reflected by its shoot and root regeneration capacities)	Antibiotic resistance: hygromycin phosphotransferase gene (*hpt*), neomycin phosphotransferase gene (*nptII)*. Fluorescence: gfp, rfp, yfp, DsRed Herbicides resistance: Bialaphos resistance gene (*bar*) and phosphinothricin acetyltransferase gene (*pat*)	An et al. (2014); Collado et al. (2016); Liu et al. (2019)

2.2.3 Stable and transient transformation

The first transgenic plant generated via *Agrobacterium*-mediated transformation approach was able to transmit its acquired T-DNA to its progenies (Zambryski et al., 1983). Ever since this first report of stable transformation, a multitude of transgenic plants with various traits have been generated (Ziemienowicz, 2014). However, generation of a true transgenic plant is laborious and time-consuming because it often requires established tissue culture techniques to regenerate a whole plant from the transformed cells or tissues (Hwang et al., 2017). Thus, stable transformation is more relevant in longer-term research of genes and for long-term production of a trait/compound in large scale (i.e. transgenic crops) (Hwang et al., 2017).

When regeneration of transgenic plants with stable inheritance of T-DNA is not necessary, transient transformation provides an alternative tool for rapid expression of the studied gene (Guo et al., 2019). It has become apparent that integration into the host cell genome was not the prerequisite for T-DNA expression (Abiri et al., 2016; Kapila et al., 1997). Due to the high copy numbers of T-DNA that was delivered into the host cells, expression level of T-DNA could be detected within hours of transformation event and usually peak within 2-5 days (Mortensen et al., 2019; Xu et al., 2014; Zheng et al., 2012). In recent years, transient transformation has progressed from just a functional tool in facilitating gene function, protein-protein interaction and protein sub-cellular localization studies for the basic research of molecular plant science to production platform for generating high value recombinant pharmaceutical proteins at an industrial scale (Dirisala et al., 2016; Moon et al., 2019; Nandi et al., 2016).

2.3 *Mucuna bracteata*

Mucuna bracteata belongs to the family of Fabaceae. It originates from Tripura, Northeast India in the Himalaya region but can also be found in the sub-tropical climate region in China, Hainan, Laos, Myanmar, Thailand, Vietnam and Andaman Island. It is an important perennial cover crop species that is widely planted as an underground cover in oil palm and rubber plantations in South India and Southeast Asia since 1990s (Figure 2.2) (Mathews, 1998; Mendham et al., 2004). *M. bracteata* has been studied for its impact on yield (Mathews & Saw, 2007; Shaharudin & Jamaluddin, 2007), nutrient return (Chiu & Madsun, 2006), biomass production and weed management for oil palm (Lee et al., 2005; Samedani et al., 2014), indicating its important role as a cover crop. It is a highly desirable cover crop mainly due to its vigorous growth characteristics, nitrogen fixation capability and high biomass. *M. bracteata* may grow to approximately 0.75 to 1.0 m per week and the mature tap roots can grow up to 2 to 4 m depth in soil (Chiu, 2007; Mathews, 1998). The leaves are large (14 cm × 10 cm) and dark green trifoliate (Mathews, 1998) (Figure 2.2C) with relatively fast leaf turnover where leaf initiation to senescence takes about 70 days (Goh & Chiu, 2007). As a legume, several *Bradyrhizobia* strains have been isolated from *M. bracteata* and their roles in nodulation induction and improving nitrogen fixation efficiency have been evaluated (Mathews et al., 2007). Its vigorous growth and ability to tolerate a wide range of climatic conditions including drought and shade conditions made *M. bracteata* a preferable cover crop over conventional cover crops, such as *Pueraria phaseoloides* and *Calapogonium mucunoides* (Figure 2.2). This is because most conventional cover crops tend to die-back after several years of establishment due to the shading effect from the growing oil palm canopies (Mathews & Saw, 2007).

Mucuna is a seed-grown annual herbaceous plant (Chattopadhyay et al., 1995). The presence of needle-like hair trichomes around the seed pods that causes great irritation when touched poses significant challenges of using seeds as starting material (Faisal et al., 2006). Moreover, low germination rate and poor viability of the seeds have limited its supply to plantation companies. Most importantly, seed production is only available to a certain climatic region in North India (Chiu 2007) as *M. bracteata* rarely produces seeds outside of this region although it may flower occasionally. Therefore, plantation companies rely heavily on seed producing companies for their constant supply of *Mucuna* seeds. This dependency is exacerbated with stringent regulatory procedure that may vary between countries for importation of this 'foreign' seeds (Chee, 2007). Although some plantation companies have developed their propagation method through stem cutting, the method is cumbersome and the success rate is very much depends on the skill of the laborers and the age of the mother stem (Lee et al., 2007). Given the challenges to obtain reliable planting material, no report has been found on its propagation through plant tissue culture technique up to this date.

Besides being a cover crop, *M. bracteata* has been found to contain L-Dopa, a direct precursor to dopamine which is used to treat Parkinson's disease (Lampariello et al., 2012; Leelambika et al., 2010). *M. bracteata* is also popular in Ayurvedic medicine for constipation, oedema, fever, delirium, and dysmenorrhea (Sangvikar et al., 2016). Recent studies found that the extracts isolated from young and mature leaves of *M. bracteata* showed anti-proliferative activity against nasopharyngeal carcinoma cells (Mai et al., 2009), whereas seed extracts demonstrated potential antimicrobial properties (Ashok et al., 2009). Despite its importance as cover crop in plantations and its medicinal prospects, there are very limited reports on biotechnological application in the study of this plant.

Figure 2.2: *Mucuna bracteata* application as cover crop in oil palm plantations.These images were photographed at an oil palm plantation located in Tangkak, Johor, Malaysia in 2019.

2.4 Toxoplasmosis

Toxoplasmosis is a highly prevalent zoonotic infection caused by the protozoan *Toxoplasma gondii*. It has been described since 1908 by Nicolle (1908) in gundi, a rodent from North Africa (Figure 2.3). It can infect humans as well as warm-blooded animals, including mammals and birds (Dubey et al., 2009). However, its primary host is cat in which the sexual parasitic cycle resides and spread oocysts through feces (Figure 2.4) (Esch & Petersen, 2013). Its transmissions to humans could be caused either by ingestion of infected meat; ingestion of soil, water or food contaminated or come into contact with infected cat feces, and transmission from infected mother to fetus (vertical transmission) (Robert-Gangneux & Dardé, 2012).

Approximately, 25 to 30% of the world's human population is infected by *Toxoplasma* (Montoya & Liesenfeld, 2004). However, the exact prevalence varies from 10 to 90% of the adult population, according to the region or food habits (Meireles et al., 2015). For instance, the prevalence of toxoplasmosis was observed to be 50-80% of women in Brazil (Dubey et al., 2012) and 30% of the general population in Malaysia (Yan et al., 2018). It has been classified as one of five Neglected Parasitic Infections (NPI) by the Centers for Disease Control and Prevention (CDC) in the United States (Yan et al., 2018). Although the disease is asymptomatic in healthy human, it may be fatal for immune-compromised patients such as HIV, organ transplant recipient, patients undergoing chemotherapy and pregnant women. An infection during pregnancy can cause congenital defects, spontaneous abortion and severe fetal abnormalities (Dubey et al., 2012).

Besides human, toxoplasmosis in animals especially livestock is also a major concern because as mentioned earlier, ingestion of infected meat is one of the main sources for infections in human. Toxoplasmosis in livestock also leads to significant economic losses especially in sheep, goats and lambs through abortions, stillbirths, early embryonic death and neonatal mortality or birth of live but weak offspring (Sander et al., 2018). Pregnancy losses in goats and sheep attributed to toxoplasmosis infections was reported to be between 0.7 and 1.4 million annually in the European Union (Katzer et al., 2011).

Currently, treatment of toxoplasmosis in humans involves prescription of several drugs (Rajapakse et al., 2017). These drugs often cause inflammatory side effects, teratogenic and have become increasingly expensive and unaffordable in less developed nations (Hernandez et al., 2017; Lim et al., 2018; Sander et al., 2018). Most importantly, continuous prescription of drugs as maintenance therapy has led to the emergence of drug-resistant *T. gondii* strains (Sims, 2009). On the other hand, treatment of toxoplasmosis in livestock animals involves vaccination to control abortions and to prevent the transmission of the parasite to humans (Wang et al., 2019). Currently, only one commercial vaccine, Toxovax®, for sheep is approved for usage in UK, New Zealand, France and Ireland (Wang et al., 2019). However, this vaccine has a relatively short shelf life, induces a short-term immunity and has a high cost of production (Sander et al., 2018). Due to these factors, immunotherapeutic intervention strategy was proposed (Cha et al., 2001; Fu et al., 2011). In order to lower the cost of production, plant-based production strategy to express these immunotherapeutic proteins have been utilized. Lim et al. (2018) has transiently expressed mouse-human chimeric antibodies in *N. benthamiana* with yields of 33-72 µg/g of plant tissue. Stable expression system through chloroplast transformation strategy in *N. tabacum* have been adopted to express two vaccine candidates for *T. gondii*. del L. Yácono et al. (2012) has stably expressed *T.*

gondii GRA4 antigen with yields of 6 µg/g of plant tissue whereas Albarracín et al. (2015) has stably expressed *T. gondii* SAG1 antigen with yields of 0.1-0.2 µg/g of plant tissue. Fusing the SAG1 to heat shock protein of *Leishmania infantum* had significantly increased the level of SAG1 antigen by up to 500-fold (Albarracín et al., 2015). Positive results from *in vitro* and *in vivo* murine model studies in these three reports have demonstrated the feasibility of plant-based production strategy to express functional pharmaceutical protein at a competitive level with conventional production platforms.

Figure 2.3: Gundi (*Ctenodactylus gundi*). From Tonge (2016). Copyright by creative commons - Attribution 4.0 (CC BY 3.0).

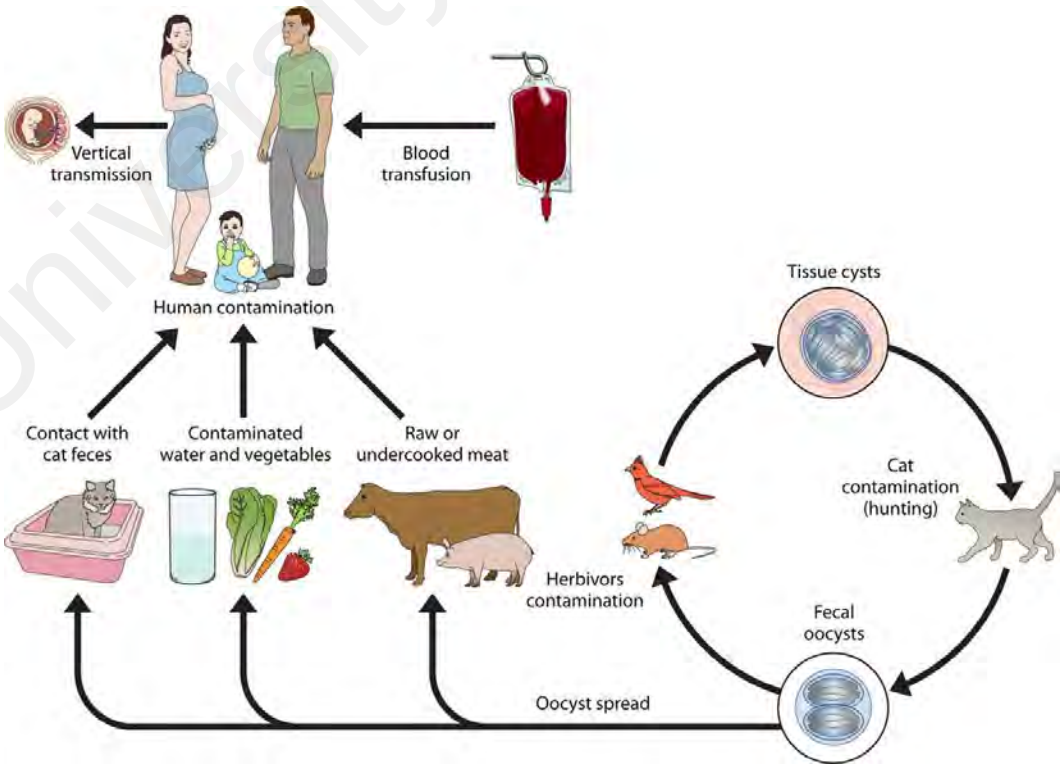


Figure 2.4: *Toxoplasma gondii* life cycle. From Esch and Petersen (2013). Copyright 2013 by the American Society for Microbiology.

CHAPTER 3: MATERIALS & METHODS

3.1 Materials

The plasmids pTRAkc-Hc (7,591 bp) (Figure 3.1) and pTRAkc-Lc (6,848 bp) (Figure 3.2) encode the heavy chain (1,371 bp) and light chain (627 bp) fragments of mouse-human chimeric anti-toxoplasma IgG (GenBank: JN104602.1), respectively (Lim et al., 2018). They were maintained in *Escherichia coli* TOP10 as glycerol stock and provided by Dr. Sherene Lim Swee Yin from Universiti Malaya, Malaysia. The vector backbone for both plasmids pTRAkc-Hc and pTRAkc-Lc did not contain selectable marker that can be used for stable transformation purpose.

The pTRA plant expression vector, pTRAkc-rfp-ERH (8,342 bp) (Figure 3.3) was provided by Prof. Dr. Rainer Fisher from Fraunhofer IME, Germany. The plasmid contains *nptII* gene as selectable marker for stable transformation purpose.

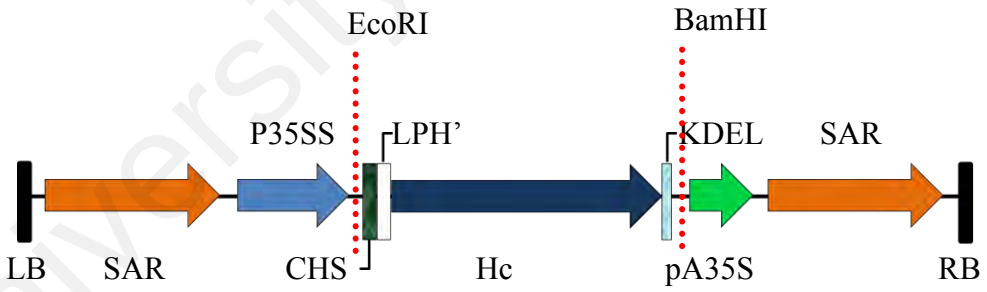


Figure 3.1: Schematic view of pTRAkc-Hc. LB, left border; SAR, scaffold attachment region of the tobacco RB7 gene (GenBank U67919); P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; EcoRI, EcoRI restriction site; CHS, chalcone synthase 5'-untranslated region; LPH, codon-optimized murine signal peptide of mAB24; Hc, heavy chain sequence of anti-toxoplasma IgG; KDEL, endoplasmic reticulum retention signal; BamHI, BamHI restriction site; pA35S, CaMV 35S polyadenylation signal; RB, right border.

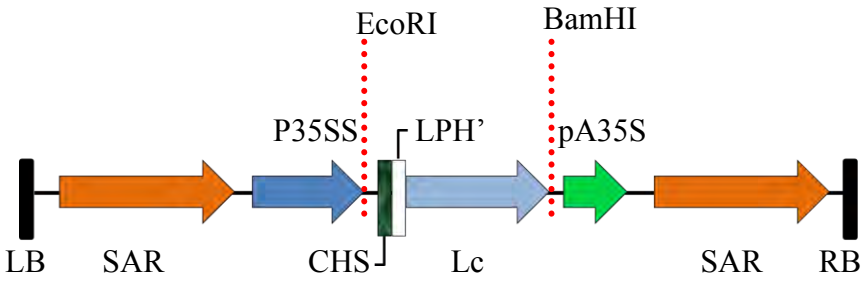


Figure 3.2: Schematic view of pTRAkc-Lc. LB, left border; SAR, scaffold attachment region of the tobacco RB7 gene (GenBank U67919); P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; EcoRI, EcoRI restriction site; CHS, chalcone synthase 5'-untranslated region; LPH, codon-optimized murine signal peptide of mAB24; Lc, light chain sequence of anti-toxoplasma IgG; BamHI, BamHI restriction site; pA35S, CaMV 35S polyadenylation signal; RB, right border.

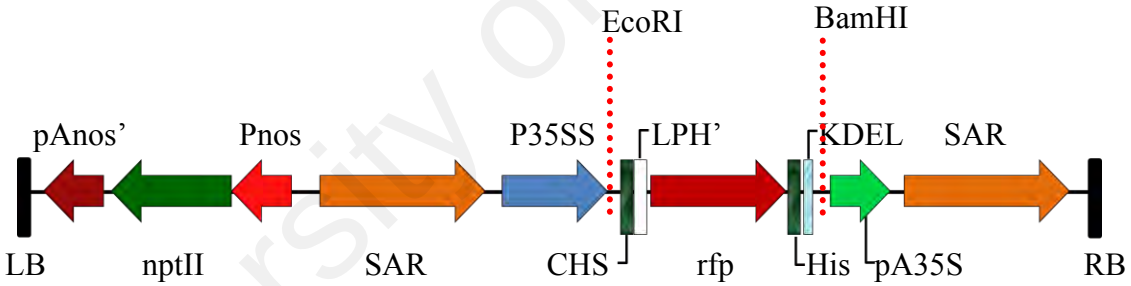


Figure 3.3: Schematic view of pTRAkc-rfp-ERH. LB, left border; pAnos', nopaline synthase gene polyadenylation signal; nptII, neomycin phosphotransferase II; Pnos, nopaline synthase promoter; SAR, scaffold attachment region of the tobacco RB7 gene (GenBank U67919); P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; EcoRI, EcoRI restriction site; CHS, chalcone synthase 5'-untranslated region; LPH, codon-optimized murine signal peptide of mAB24; rfp, red fluorescence protein from *Discosoma* sp.; pA35S, CaMV 35S polyadenylation signal; His, his tag sequence; KDEL, endoplasmic reticulum retention signal; BamHI, BamHI restriction site; RB, right border.

3.2 General molecular technique

3.2.1 DNA extraction using DNeasy Plant Mini Kit

Genomic DNA was extracted from plant tissue using DNeasy Plant Mini Kit (QIAGEN, USA) according to the manufacturer's instruction. All the buffers and reagents used were provided in the kit unless otherwise noted.

Lyophilized plant tissue was pulverized to fine powder in the presence of liquid nitrogen. 400 µL of Buffer AP1 and 4 µL of RNase A stock solution (100 mg/mL) were added to 100 mg of the powdered plant tissue. The mixture was vortex vigorously and incubated at 65 ^{o}C for 10 min. During incubation, the tube was inverted 2-3 times. Afterward, 130 µL of Buffer P3 was mixed with the lysate and incubated on ice for 5 min. The lysate was then centrifuged at 20,000 ×g for 5 min. The supernatant was transferred into QIAshredder Mini spin column and centrifuged at 20,000 ×g for 2 min. Without disturbing the cell-debris pellet, the flow-through fraction was transferred into a new microcentrifuge tube and mixed with 1.5 volumes of Buffer AW1. The mixture was transferred into DNeasy Mini spin column and centrifuged at 10,000 ×g for 1 min. The column was rinsed twice by adding 500 µL of Buffer AW2 to the column and centrifuged at 10,000 ×g for 1 min. Centrifugation was repeated at 20,000 ×g for 2 min to ensure complete removal of residual Buffer AW2. Afterward, the spin column was transferred into a clean microcentrifuge tube where 50 µL of preheat (65 ^{o}C) Buffer AE was aliquoted directly to the center of the column matrix. The column was incubated at room temperature for 5 min. Finally, the column was centrifuged at 10,000 ×g for 1 min to elute the DNA. The extracted DNA was kept in -20 ^{o}C until analysis.

3.2.2 DNA extraction using Edwards method

The protocol to extract crude genomic DNA from plant tissue was modified from Edwards et al. (1991). This method was used for crude genomic DNA extraction from large number of samples. Briefly, 20 mg of plant tissue was ground with chilled pellet pestle in the presence of 200 µL of extraction buffer (Appendix C1). Once the plant tissue macerated another 200 µL of extraction buffer (Appendix C1) was added, followed by centrifugation at 13,000 rpm for 1 min at 4 ^{o}C. The supernatant was transferred into a new tube, mixed with 1 volume of ice cold isopropanol and incubated on ice for at least 30 min. The mixture was centrifuged at 13,000 rpm for 5 min at 28 ^{o}C and the supernatant was discarded. The pellet was washed with 700 µL of 70% (v/v) ethanol and recentrifuged at 13,000 rpm for 5 min at 28 ^{o}C. The resultant pellet was air dried and resuspended in 50 µL of preheat (65 ^{o}C) Buffer AE (Section 3.2.1). The extracted DNA was kept in -20 ^{o}C until analysis.

3.2.3 Plasmid extraction

Plasmid DNA was extracted from bacterial cell culture using *EasyPure*® Plasmid MiniPrep Kit (TransGen Biotech, China) according to the manufacturer's instruction. All the buffers and reagents used were provided in the kit unless otherwise noted.

Overnight bacterial suspension culture (1 mL) was pelleted at 10,000 ×g for 1 min. The pellet was resuspended in 250 µL of Resuspension Buffer, followed by 250 µL of Lysis Buffer and 350 µL of Neutralization Buffer. The lysate was incubated at room temperature for 2 min and centrifuged at 12,000 ×g for 5 min. The supernatant was transferred into a spin column provided with the kit and centrifuged at 12,000 ×g for 1 min. The column was rinsed by adding 650 µL of Washing Buffer to the column and centrifuged at 12,000 ×g for 1 min. Centrifugation was repeated at 12,000 ×g for 2 min to ensure complete removal of residual Washing Buffer. Afterward, the spin column was transferred into a clean microcentrifuge tube where 50 µL of preheat (65 ^{o}C) Elution Buffer was aliquoted directly to the center of the column matrix. The column was incubated at room temperature for 4 min. Finally, the column was centrifuged at 10,000 ×g for 1 min to elute the plasmid DNA. The extracted plasmid was kept in -20 ^{o}C until analysis.

3.2.4 Amplification of target gene using NEXpro™ e Taq DNA Polymerase

Amplification was carried out using NEXpro™ e Taq DNA Polymerase Kit (Genes Laboratories, South Korea) according to the manufacturer's instruction. All the buffers and reagents used were provided in the kit unless otherwise noted.

Amplification was carried out in a 25 µL reaction volume containing 2.5 µL of 10× PCR buffer, 1 µM of each forward and reverse primer, 1 mM of dNTP mix and 0.125 U of NEXpro™ eTaq Polymerase. The amplification reaction was carried out in a thermocycler (Biometra, Germany) with the following cyclic profiles: initial denaturation at 95 ^{o}C for 3 min, followed by 35 cycles of 20 s denaturation at 95 ^{o}C, 20 s annealing at different temperatures based on the primer (Appendix B), 1 min extension at 72 ^{o}C and a final extension cycle at 72 ^{o}C for 5 min.

3.2.5 Amplification of target gene using *i*-Taq™ DNA Polymerase

Amplification was carried out using *i*-Taq™ DNA Polymerase Kit (iNtRON, South Korea) according to the manufacturer's instruction. All the buffers and reagents used were provided in the kit unless otherwise noted.

Amplification was carried out in a 25 µL reaction volume containing 2.5 µL of 10× PCR buffer, 2.5 mM of $MgCl_2$, 1 µM of each forward and reverse primer, 1 mM of dNTP mix and 0.05 U of *i*-Taq DNA polymerase. The amplification reaction was carried out in a thermocycler (Biometra, Germany) with the following cyclic profiles: initial denaturation at 94 ^{o}C for 2 min, followed by 35 cycles of 30 s denaturation at 94 ^{o}C, 30 s annealing at different temperatures based on the primer (Appendix B), 30 s extension at 72 ^{o}C and a final extension cycle at 72 ^{o}C for 5 min.

3.2.6 Amplification of target gene using Q5® Hot Start High-Fidelity DNA Polymerase

Amplification was carried out using Q5® Hot Start High-Fidelity DNA Polymerase Kit (New England Biolabs, USA) according to the manufacturer's instruction. All the buffers and reagents used were provided in the kit unless otherwise noted.

Amplification was carried out in a 25 µL reaction volume containing 5 µL of 5× Q5 Reaction buffer, 0.5 µM of each forward and reverse primer, 200 µM of dNTP mix and 0.5 U of Q5 Hot Start High-Fidelity DNA polymerase. The amplification reaction was carried out in a thermocycler (Biometra, Germany) with the following cyclic profiles: initial denaturation at 98 ^{o}C for 30 s, followed by 35 cycles of 10 s denaturation at 98 ^{o}C, 20 s annealing at different temperatures based on the primer (Appendix B), 1 min extension at 72 ^{o}C and a final extension cycle at 72 ^{o}C for 2 min.

3.2.7 Analysis of DNA via gel electrophoresis

Agarose gel 1% (w/v) was prepared by mixing 0.2 g of agarose gel (Vivantis, USA) with 20 mL of 1× TBE (Appendix C5). The mixture was heated on high heat in microwave for about 2 min or until all the agarose has dissolved. The mixture was then cooled down under running tap water and mixed with 1 µL of ethidium bromide. The agarose gel was casted in a gel tray with the well comb in place. Once the agarose gel has solidified (usually after 15 min), the agarose gel was placed into a gel box and immersed in 1× TBE (Appendix C5).

For genomic DNA and PCR product, sample was prepared by mixing 5 µL of DNA product with 1 µL of 6× Loading Buffer RED (EURx, Poland). The prepared sample was then loaded into the well of the gel. For restriction digest product (Section 3.2.8), 5 µL of the digested product was loaded directly into the well of the gel. 3 µL of Perfect Plus™ 1 kb DNA Ladder (EURx, Poland) was loaded as molecular weight ladder. The samples were electrophorased at 110V until the front dye was approximately 80% of the way down the gel. The gel was then viewed and analyzed using Gel-Pro® Imager and Analyzer (MicroLAMBDA, USA).

3.2.8 Restriction digests using FastDigest restriction enzymes

Restriction digest of DNA sample (plasmid DNA or PCR product) was carried out using FastDigest restriction enzymes (Thermo Scientific, USA) according to the manufacturer's instruction. All the buffers and reagents used were provided in the kit unless otherwise noted.

The reaction components were combined at room temperature in the order indicated in Table 3.1.

Table 3.1: Reaction components of restriction digest of different type of DNA

Component	Plasmid DNA	PCR product
dH_2O	15 µL	17 µL
10× FastDigest Green Buffer	2 µL	2 µL
DNA	2 µL (up to 1 µg)	10 µL (~0.2 µg)
FastDigest enzyme	1 µL	1 µL
Total volume	20 µL	30 µL

If the DNA was digested with two types of restriction enzyme (double digestion), the combined volume of the enzymes in the reaction mixture was ensured not to exceed 1/10 of the total reaction volume. The reaction mixture was mixed gently, spun down and incubated at 37 ^{o}C in a water bath for 1 h. The reaction mixture was then incubated at 80 ^{o}C in a heat block to inactivate the enzyme. Digestion efficiency was evaluated via gel electrophoresis (Section 3.2.7). Based on the gel electrophoresis image, digestion was deemed successful with the appearance of lower molecular weight bands and the absence of initially higher molecular weight bands. An unchanged band pattern indicated an unsuccessful digestion. Band containing the DNA fragment of interest was gel extracted and purified (Section 3.2.9).

3.2.9 Gel extraction and purification

DNA fragment of interest analyzed via gel electrophoresis (Section 3.2.7) was extracted and purified using QIAquick Gel Extraction Kit (QIAGEN, USA) according to the manufacturer's instruction. All the buffers and reagents used were provided in the kit unless otherwise noted.

DNA fragment was excised from the agarose gel (Section 3.2.7) with a clean, sharp scalpel and transferred into a microcentrifuge tube. In the tube, 3 volumes of Buffer QG were added to 1 volume of gel (100 mg of agarose gel equal roughly to 100 µL). The mixture was incubated at 50 ^{o}C for 10 min or until the gel slice has completely dissolved. The mixture was vortex every 2-3 min during incubation. Solubilized agarose was mixed with 1 gel volume of isopropanol, transferred into QIAquick spin column and centrifuged at 17,900 ×g for 1 min. The column was rinsed by adding 750 µL of Buffer PE to the column and centrifuged at 12,000 ×g for 1 min. Centrifugation was repeated at 17,900 ×g for 2 min to ensure complete removal of residual Buffer PE. Afterward, the spin column was transferred into a clean microcentrifuge tube where 30 µL of preheat (65 ^{o}C) Buffer EB was aliquoted directly to the center of the column matrix. The column was incubated at room temperature for 4 min. Finally, the column was centrifuged at 17,900 ×g for 1 min to elute the purified DNA. The purified DNA was kept in -20 ^{o}C until analysis.

3.2.10 Ligation of insert DNA into plasmid vector DNA

Ligation of insert DNA containing the target gene into plasmid vector DNA was carried out using Rapid DNA Ligation Kit (Thermo Scientific, USA) according to the manufacturer's instruction. All the buffers and reagents used were provided in the kit unless otherwise noted.

The ligation mixture was prepared by combining the reaction components indicated in Table 3.2 on ice.

Table 3.2: Reaction component of ligation mixture

Component	Amount
Linearized plasmid DNA	10-100 ng
Insert DNA (at 3:1 molar excess over vector)	Variable
5× Rapid Ligation Buffer	4 µL
T4 DNA Ligase, 5 U/µL	1 µL
dH_2O	Up to 20 µL
Total volume	20 µL

The reaction mixture was vortex, spun down and incubated at 22 ^{o}C in a water bath for 1 h. Ligation efficiency was evaluated via gel electrophoresis (Section 3.2.7). Based on the gel electrophoresis image, ligation was deemed successful with the appearance of higher molecular weight bands and decreased intensity of the vector and insert bands. An unchanged band pattern indicated an unsuccessful ligation. Following successful ligation, 5 µL of the ligation mixture was used for bacterial transformation (Section 3.2.11)

3.2.11 Introduction of plasmid DNA into NEB 5-alpha F' I^q Competent *E. coli*

A tube containing 50 µL of NEB 5-alpha F' I^q Competent *E. coli* cells (Thermo Scientific, USA) was thawed on ice or until the last ice crystals disappeared. Up to 5 µL of plasmid DNA was added to the cell. The tube was carefully flicked to mix the cells and DNA. The mixture was then incubated on ice for 30 min and heat shock at 42 °C for 30 sec. The cells were incubated on ice for 5 min and resuspended in 950 µL of room temperature SOC media (Appendix C12). The cell culture was incubated at 37 °C for 1 h with agitation of 250 rpm. The culture was then spread onto LB selection plate (Appendix C6) and incubated overnight at 37 °C. Selected colonies were picked for PCR colony analysis (Section 3.2.14).

3.2.12 Preparation of *Agrobacterium tumefaciens* competent cells

Primary culture of *A. tumefaciens* was setup by culturing 100 µL of -80 °C glycerol stock (Section 3.2.15) in 10 mL of YEB media supplemented with appropriate antibiotic based on the strains of *A. tumefaciens* used (Appendix C7). The culture was incubated at 28 °C in dark condition with agitation of 120 rpm for overnight. Secondary culture was setup by subculturing 100 µL of the primary culture into 10 mL of YEB media supplemented with appropriate antibiotic based on the strains of *A. tumefaciens* used (Appendix C7). The culture was incubated under similar culture conditions until the OD_{600} reached ~0.5. The culture was aliquot into microcentrifuge tube, 1 mL each tube, chilled on ice for 10 min and pelleted by centrifugation at 3,000 ×g for 5 min at 4 °C. The pellet was then resuspended in 100 µL of ice cold 20 mM $CaCl_2$ (Appendix C8) and 20 µL of ice cold 100% glycerol. Resuspended culture was snap frozen in liquid nitrogen and kept in -80 °C.

3.2.13 Introduction of plasmid DNA into *Agrobacterium tumefaciens* competent cells

A tube containing 100 µL of competent *A. tumefaciens* cells (Section 3.2.12) was thawed on ice or until the last ice crystals disappeared. Up to 10 µL of plasmid DNA was added to the cell mixture and the tube was carefully flicked to mix the cells and DNA. The mixture was then incubated on ice for 30 min, snap frozen in liquid nitrogen and heat shock at 37 ^{o}C for 4 min. The cells were incubated on ice for 5 min and resuspended in 1 mL of room temperature YEB media (Appendix C7). The cells were incubated at 28 ^{o}C for 4 hours with gentle agitation. After incubation, the cells were pelleted at 12,000 rpm for 2 min and resuspended in 50 µL of YEB broth. The culture was then spread onto appropriate YEB selection plate based on the strains of *A. tumefaciens* used (Appendix C7). The culture was incubated at 28 ^{o}C up to 4 days. Selected colonies were picked for PCR colony analysis (Section 3.2.14).

3.2.14 PCR colony of transformed cells harboring introduced plasmid

Bacterial colonies (Section 3.2.11) (Section 3.2.13) were picked using sterile tips, touched lightly into the inner side of PCR tube and streaked onto appropriate selection plate based on the bacterial species and strain used (Appendix C6) (Appendix C7) to establish "plate library". The plate was incubated in the dark at 28 ^{o}C for *A. tumefaciens* and at 37 ^{o}C for *E. coli*. PCR was carried out as described in Section 3.2.4 and analyzed as described in Section 3.2.7.

3.2.15 Preparation of bacterial glycerol stock for -80 °C storage

Bacterial glycerol stock was prepared by mixing 800 µL of bacterial suspension with 200 µL of 100% glycerol. Then, the mixture was snap frozen in liquid nitrogen and kept in -80 °C.

3.2.16 Bacterial suspension culture

For *E. coli* culture, 10 mL of LB broth (Appendix C22) supplemented with 100 µg/mL of ampicillin (Appendix C16) was inoculated with either bacterial colony from "plate library" (Section 3.2.14) using sterile tips or 100 µL of bacterial glycerol stock (Section 3.2.15). The culture was incubated at 37 °C in dark condition with agitation of 120 rpm for overnight.

For *A. tumefaciens* culture, 10 mL of YEB broth supplemented with appropriate antibiotic based on the strain and plasmid harbored (Appendix C7) was inoculated with either bacterial colony from "plate library" (Section 3.2.14) using sterile tips or 100 µL of bacterial glycerol stock (Section 3.2.15). The culture was incubated at 28 °C in dark condition with agitation of 120 rpm for overnight.

3.2.17 Sequencing analysis

Sequencing results of plasmid/purified PCR product (Genomics Biosci & Tech. Co., Ltd., Taiwan) were analyzed using FinchTV Software (Geospiza, UK) and BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequence alignment was performed using ClustalW Multiple Alignment analysis (BioEdit Sequence Alignment Editor, US).

3.3 General tissue culture techniques

3.3.1 Sources of plant materials

Seeds of *M. bracteata* DC. ex Kurz was procured from Chemiseed Sdn Bhd (https://chemiseed.com), a local seed supplier in Subang Jaya, Selangor, Malaysia. Seeds of *N. tabacum* cv. SR1 and *N. benthamiana* were obtained from Plant Biotechnology Research Lab, Institute of Biological Sciences, Faculty of Science, Universiti Malaya, Malaysia.

3.3.2 *In vitro* culture of *Mucuna bracteata*

Seeds of *M. bracteata* were cleaned thoroughly by rinsing twice with tap water, each time for at least 2 min, followed by distilled water for 10 min and dried at room temperature for two days. Sixty-five dry seeds were immersed in 100 mL of concentrated sulfuric acid (98%) for scarification (Section 3.5.1) and rinsed at least five times with sdH_2O. Scarified seeds were surfaced sterilized (Section 3.5.2) and primed (Section 3.5.3) before germination (Section 3.5.4).

3.3.3 *In vitro* culture of *Nicotiana* species

Seeds of *Nicotiana* species were surface sterilized by gentle agitation in 70 % (v/v) ethanol for 1 min, rinsed once with sdH_2O and gently agitated in 25 % (v/v) Clorox® for 15 min. The seeds were then rinsed at least six times with sdH_2O and cultured on MS media (Appendix D1) for germination in growing condition as described in Section 3.3.4.

3.3.4 *In vitro* culture growing condition

Unless otherwise noted, all *in vitro* cultures were maintained under 16 h light regime with a light intensity of 31.4 μmol m^{-2} s^{-1} provided by cool fluorescent lamps in 25 ± 3 ^{o}C environment.

3.3.5 Acclimatization of *in vitro* culture plantlet

Rooted plantlets were acclimatized at 25-30 ^{o}C environments with 60 % humidity level in Plant Biotech Facility, Universiti Malaya. The roots were washed with distilled water before the plantlets were transferred to polybags containing sterilized garden soil. The plants were fully covered with perforated transparent plastic bags before gradually removed after two weeks of culture. The plants were watered twice a day. Fertilizers were applied once for *M. bracteata* after 20 days of planting and twice for *N. benthamiana* and *N. tabacum* cv. SR1 after 14 days and 28 days of planting.

3.4 General protein analysis techniques

3.4.1 Samples collection

Harvested plant tissue was snap frozen in liquid nitrogen and kept in -80 ^{o}C until use.

3.4.2 Total soluble protein extraction

Lyophilized leaves were pulverized to fine powder in the presence of liquid nitrogen. Total soluble protein (TSP) was extracted from the powdered leaves in two volumes of phosphate buffered saline (PBS) (Amresco, USA) or NEB (Appendix E4). The crude extract was clarified by centrifugation at 13,000 rpm for 20 min at 4 ^{o}C. The supernatant was recentrifuged for 20 min at 4 ^{o}C. The final supernatant was kept at 4 ^{o}C for immediate use.

3.4.3 Total soluble protein quantification

Total soluble protein (TSP) was quantified using Quick Start™ Bradford Protein Assay (Bio-Rad, USA) according to the manufacturer's instruction. All the buffers and reagents used were provided in the kit unless otherwise noted.

Standard curve was constructed using bovine serum albumin (BSA) (VWR Life Science, USA) dissolved in PBS (Amresco, USA) or NEB (Appendix E4). The standard set was prepared as indicated in Table 3.3.

Table 3.3: Preparation of standard curve set

Tube	Standard volume (µL)	Source of standard	Diluent volume (µL)	Final protein concentration (µg/mL)
1	70	2 mg/mL BSA stock	0	2,000
2	75	2 mg/mL BSA stock	25	1,500
3	70	2 mg/mL BSA stock	70	1,000
4	35	Tube 2	35	750
5	70	Tube 3	70	500
6	70	Tube 5	70	250
7	70	Tube 6	70	125
8 (blank)	-	-	70	0

20 µL of each standard or sample was mixed with 1 mL of 1× dye reagent in separate clean cuvette. The mixture was incubated for at least 5 min in the dark at room temperature. Absorbance at 595 nm was measured using spectrophotometer (Eppendorf, Germany). The unknown TSP concentration in the sample was then determined based on the standard curve.

3.4.4 Anti-toxoplasma IgG purification

Anti-toxoplasma IgG in 400 µg of TSP (Section 3.4.3) was purified using NAb™ Protein A spin column (Thermo Scientific, USA) according to the manufacturer's instructions.

Following the manufacturer's protocol, the column was equilibrated twice, each time by dispensing 400 µL of Binding Buffer (Appendix E5) into the column and centrifuged at 5,000 ×g for 1 min. Sample containing 400 µg of TSP was added to the column and incubated at room temperature with agitation of 700 rpm using Eppendorf ThermoMixer® (Eppendorf, Germany) for 15 min. Non-bound sample was removed from the column by centrifugation at 5,000 ×g for 1 min. The column was rinsed three times, each time by adding 400 µL of Binding Buffer (Appendix E5) to the column and centrifuged at 5,000 ×g for 1 min. Then, the column was transferred to a clean 2 mL microcentrifuge tube containing 40 µL of Neutralization Buffer (Appendix E6). Bound antibody in the column was eluted out by adding 400 µL of Elution Buffer (Appendix E7) to the column and centrifuged at 5,000 ×g for 1 min. Elution into a new 2 mL microcentrifuge tube containing 40 µL of Neutralization Buffer (Appendix E6) was repeated two times to obtain three elution fractions.

3.4.5 Anti-toxoplasma IgG quantification

Concentration of anti-toxoplasma IgG in the eluate fractions from NAb™ Protein A spin column (Thermo Scientific, USA) (Section 3.4.4) was quantified by measuring the absorbance at 280 nm (Abs. 280) using nanophotometer (Implen, Germany) followed by calculation according to the formula provided by the manufacturer. The formula describing the concentration to absorbance relation is a modification of the Lambert-Beer equation.

$$C_{prot.} = \text{Abs. 280} * \text{A280 factor} * \text{lid factor} * \text{dilution factor}$$

C_{prot} : protein concentration (mg/mL)

Abs. 280 : absorbance (AU) of proteins at 280 nm

A280 factor: molecular weight $_{prot.}$ / molar extinction coefficient ($M^{-1}*cm^{-1}$) $_{prot.}$

Lid factor : dependent on the used dilution lid

In this calculation, the A280 factor was 0.71 for IgG.

A280 factor	: 0.71
Molecular weight (g/mol)	: 150,000
Molar extinction coefficient ($M^{-1}*cm^{-1}$)	: 210,000

The lid factor which was dependent on the used dilution lid was 10.

Mixture of 400 µL Binding buffer (Appendix E5) and 40 µL Neutralization buffer (Appendix E6) was used as a blank for the measurements.

3.4.6 Concentrating anti-toxoplasma IgG and buffer exchange

The eluate fractions from NAb™ Protein A spin column (Thermo Scientific, USA) (Section 3.4.4) were concentrated and buffer exchanged with PBS (Amresco, USA) using Pierce Protein Concentrator, PES 3K (Thermo Scientific, USA) according to the manufacturer's instructions. For both procedures, the samples were centrifuged at 5,000 × g and 25 ^{o}C until the retentate volume was less than 10% of the original volume.

3.4.7 Western blot analysis

The purified and concentrated samples (Section 3.4.6) were boiled at 100 ^{o}C for 5 min in a 1:1 ratio of samples and 2× loading sample buffer (Appendix E8). The samples were centrifuged at 17,000 × g for 2 min prior to loading on 4% stacking (Appendix E9) and 12% separating (Appendix E10) SDS-polyacrylamide gel (SDS-PAGE). The samples were electrophorased at 80 V under reducing conditions until the front dye reached the end of the gel. The gel was rinsed twice with dH_2O and blotted onto Amersham™ ECL™ Sensitive Nitrocellulose membrane (GE Healthcare, USA) using trans-blot Mini PROTEAN (Bio-Rad, USA). The transfer was performed using FlashBlot Transfer Buffer (Advansta, USA) for 30 min at 55 V according to the manufacturer's instruction. The membrane was rinsed twice by gentle agitation with ultrapure water for a total of 10 min and blocked for an hour at room temperature with Blocking buffer (Appendix E11) and 30 rpm agitation. Afterward, the membrane was washed with TBST (Appendix E12) for 5 min at room temperature with 80 rpm agitation. Washing was repeated for a total of three times.

After the final wash, the membrane was statically incubated overnight at 4 ºC in 10 mL of primary antibody solution (Appendix E13) containing monoclonal anti-human IgG Fab fragment antibody (Cat number: ab771) (Abcam, UK) diluted 1:1,000. Then, the membrane was washed again three times with TBST (Appendix E12) for 5 min at room temperature with agitation of 80 rpm each time. After the final wash, the membrane was incubated at room temperature in dark condition for 2 h with agitation of 40 rpm in 10 mL of secondary antibody solution (Appendix E14) containing polyclonal goat anti-mouse IgG Fc conjugated to horse-radish peroxidase (HRP) (Cat number: ab20043) (Abcam, UK) diluted 1:5,000. The membrane was washed three times with TBST (Appendix E12) for 5 min at room temperature agitation of 80 rpm each time. Detection was developed using the WesternBright^{TM} Quantum Chemiluminescent HRP Substrate kit (Advansta, USA) according to the manufacturer's instruction and imaged using Bio-Rad Chemidoc MP system (Bio-Rad, USA).

3.4.8 ELISA

The antigen binding properties of the purified and concentrated samples (Section 3.4.6) was analyzed by using Human Anti-*Toxoplasma gondii* IgG ELISA Kit (Abcam, UK) according to the manufacturer's instruction. All the buffers and reagents used were provided in the kit unless otherwise noted.

Following the manufacturer's protocol, all samples were diluted 1:100 (v/v) with IgG Sample Diluent. 100 µL of each standard and diluted sample were loaded into appropriate wells. One well was left for substrate blank purpose. The content from each well was aspirated after incubated at 37 ^{o}C for 1 h and the wells were washed three times with 300 µL of 1× washing solution. Washing was done carefully to avoid spilling over into neighboring wells. The soaked time between each wash cycle was ensured to be more than 5 seconds. After the last wash, the remaining 1× washing solution was removed by aspiration. The plate was inverted and blotted against clean paper towels to remove excess liquid. 100 µL of *Toxoplasma gondii* anti-IgG HRP conjugate was added into all wells except for the blank well. The wells were incubated for 30 min at room temperature in dark condition. Washing step was repeated before adding 100 µL of TMB substrate solution into all wells and incubated at room temperature for 15 min in dark condition. 100 µL of Stop solution was added into all wells in the same order and at the same rate as previously for the TMB substrate solution. Absorbance was measured at 450 nm within 30 min after the addition of Stop solution using microplate reader (Tecan, Switzerland).

3.5 *In vitro* propagation of *Mucuna bracteata*

3.5.1 Seeds scarification

During scarification process in Section 3.3.2, the seeds were immersed in 100 mL of concentrated sulfuric acid (98%) and agitated at 100 rpm for 10, 30 or 60 min. Seeds without immersion in concentrated sulfuric acid (98%) were considered as control. All scarified seeds were rinsed at least five times with sdH_2O before culturing on MS media (Appendix D1). The cultures were maintained in dark condition at room temperature.

3.5.2 Seeds surface sterilization

During surface sterilization procedure in Section 3.3.2, three surface sterilization protocols were evaluated on seeds that have been scarified for 30 min in sulfuric acid (Section 3.5.1). In the first treatment, scarified seeds were immersed in 95% (v/v) ethanol for 1 min followed by rinsing three times with sdH_2O. In the second treatment, scarified seeds were immersed in 50% (v/v) Clorox® (commercial bleach with 5.25% (w/v) sodium hypochlorite) containing 7 drops of Tween 20 per 100 mL and agitated at 100 rpm for 10 min. The seeds were then rinsed five times with sdH_2O. In the third treatment, scarified seeds were immersed in 95% (v/v) ethanol for 1 min, rinsed three times with sdH_2O, disinfected in 50% (v/v) Clorox® containing 7 drops of Tween 20 per 100 mL for 10 min and washed five times with sdH_2O. Seeds without surface sterilization treatment were considered as control. All disinfected seeds were rinsed once with sdH_2O before culturing on MS media (Appendix D1). The cultures were maintained in dark condition at room temperature.

3.5.3 Seeds priming

During seeds priming process in Section 3.3.2, seeds that have been scarified for 30 min in sulfuric acid (Section 3.5.1) without any surface sterilization treatment (Section 3.5.2) were primed in two ways; hydropriming and osmopriming.

In hydropriming, seeds that have been scarified for 30 min in sulfuric acid (Section 3.5.1) were rinsed at least five times with sdH_2O and imbibed in sdH_2O for different durations (0-48 h). The imbibed seeds were rinsed once with sdH_2O before culturing on double layers of cotton roll moistened with different volumes of sdH_2O (0-40 mL). The suitable imbibition period and volume of sdH_2O selected from the hydropriming experiment was used for osmopriming treatment.

In osmopriming, seeds that have been scarified for 30 min in sulfuric acid (Section 3.5.1) were rinsed at least five times with sdH_2O and imbibed in either 10% (w/v) polyethylene glycol (PEG) 6000 solution (Appendix D2) or sdH_2O for 6 h. Seeds imbibed in 10% (w/v) PEG 6000 solution (Appendix D2) were rinsed at least five times with sdH_2O before culturing on double layers of cotton roll moistened with 10 mL of sdH_2O, whereas seeds imbibed in sdH_2O were cultured on double layer cotton roll moistened with 10 mL of sdH_2O, 10 mL of 2.27 mM ascorbic acid (Appendix D3) or 10 mL of 0.1% (w/v) activated charcoal (Appendix D4). The cultures were maintained in dark condition at room temperature.

The percentage of seed germination and the length of seedling were recorded at 12 days post culture. Seed vigor index was then calculated according to the following formula (Abdul-Baki & Anderson, 1973):

Seed vigor index = Germination (%) × Seedling length at 12 days post culture (mm)

3.5.4 Seeds germination conditions

During germination procedure in Section 3.3.2, seeds that have been scarified for 30 min in sulfuric acid (Section 3.5.1) without any surface sterilization treatment (Section 3.5.2) and imbibed for 6 h in sdH_2O (Section 3.5.3) were germinated on either MS media (Appendix D1) or double layers of cotton roll moistened with 10 mL of sdH_2O. All cultures were maintained at room temperature either in dark condition or under 16 h light regime (Section 3.3.4).

3.5.5 Shoot multiplication

Nodal segments, leaf discs (50 mm diameter) and cotyledonary nodes were used as explants for shoot multiplication. Nodal segments and leaf discs were excised from 1 month old *in vitro* seedlings (Figure 3.4: left panel) (Section 3.3.2), whereas cotyledonary nodal explants were prepared from 5 days old seed cultures by removing the plumules and radicles (Figure 3.4: right panel) (Section 3.3.2). All explants were cultured on MS media (Appendix D1) supplemented with different concentrations of 6-benzylaminopurine (BAP) (4.44–17.76 µM) (Appendix D10) (Duchefa Biochemie, Netherlands) and kinetin (4.65–18.59 µM) (Appendix D12) (SIGMA, USA). All cultures were maintained in growing condition as described in Section 3.3.4. The shoot height and number of shoots per nodal segment and cotyledonary node were recorded after one month of culture. The percentage of callus formation in leaf discs was determined after two months of culture since there was no shoot formation.

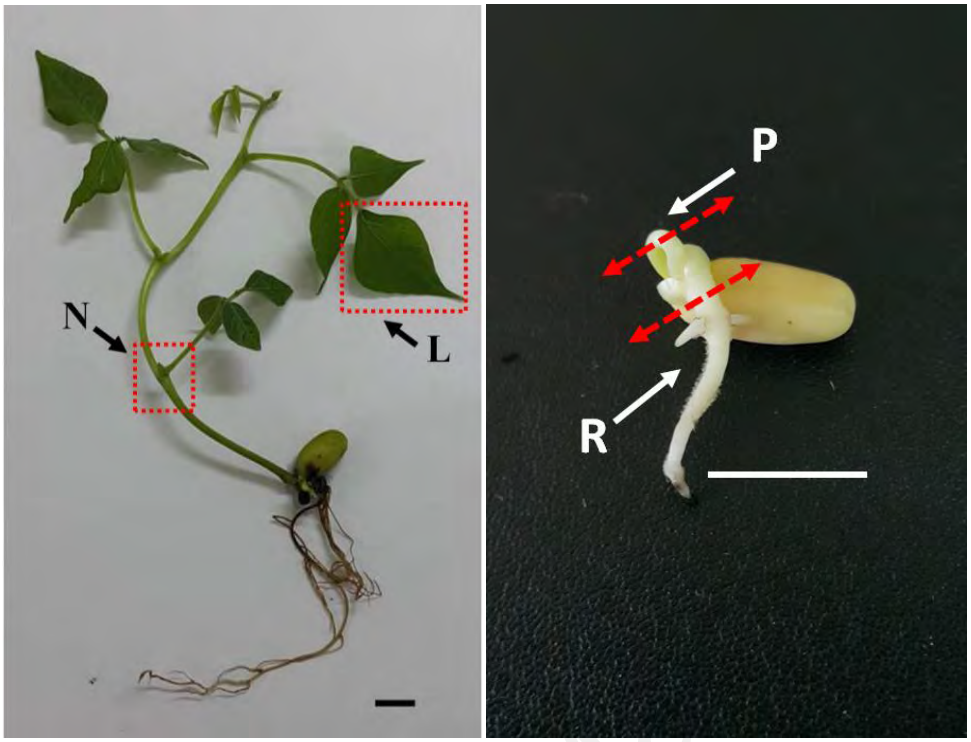


Figure 3.4: Source of explants for shoot induction. Left panel: Leaf discs (L) and nodal segments (N) from 1 month old *in vitro* seedling. Right panel: The plumule (P) and radicle (R) were excised from 5 days old *in vitro* germinating seed for cotyledonary node explants. Bar = 1 cm.

3.5.6 Root induction

Elongated shoots (5-20 cm in length) were excised from shoot clumps (Figure 3.5) (Section 3.5.5) and transferred to MS media (Appendix D1) supplemented with different concentrations of 1-naphthylacetic acid (NAA) (2.69–16.1 µM) (Appendix D11) (Duchefa Biochemie, Netherlands). All cultures were maintained in growing condition as described in Section 3.3.4. The percentage of shoots with root formation, number of roots per shoot and root length were recorded after one month of culture.

Figure 3.5: Shoot clump generated from shoot multiplication (Section 3.5.5). Bar = 1 cm.

3.5.7 Simple sequence repeats (SSR) analysis

Genomic DNA was extracted (Section 3.2.1) from leaves of two seed-germinated plants (Section 3.3.2) and ten randomly selected *in vitro*-derived plants (Section 3.5.5). The extracted genomic DNA was used as DNA template for amplification (Section 3.2.5) using ten SSR primers (Appendix B: Table 2). The PCR products were analyzed as described in Section 3.2.7.

3.5.8 DNA content and genome size estimation

A young reddish-purple leaf (one week-old) randomly selected from *in vitro*-derived (Section 3.5.5) or seed-germinated plants (Section 3.3.2) was chopped with razor blades in a Petri dish containing 0.9 mL of LB01 lysis buffer (Appendix D5) that was supplemented with 50 µg/mL RNase A and 50 µg/mL propidium iodine to release nuclei (Doležel & Bartoš, 2005). The suspension was filtered through a 40 µm Falcon™ cell strainer (Thermo Scientific, USA) into a 5 mL round bottom centrifuge tube. The filtrate was incubated at 4 ºC for 10 min. The suspension was analyzed using BD FACSCalibur (BD Bioscience, USA). A minimum of 10,000 nuclei were measured per sample. Three randomly selected *in vitro*-derived (Section 3.5.5) and seed-germinated plants (Section 3.3.2) with a total of three young leaves per plant were used in this study.

The histograms of relative fluorescence intensity from *in vitro*-derived plants were compared with seed-germinated plants to determine the changes of DNA content based on the following formula (Doležel & Bartoš, 2005):

$$\text{DNA content (pg)} = \frac{\text{Fluorescence intensity of sample}}{\text{Fluorescence intensity of standard}} \times \text{Genome size of soybean (2.5 pg)}$$

Fluorescence intensity of nuclei extracted from young leaves of soybean (*Glycine max* cv. Polanka) was used as the standard. Based on the calculated DNA content, genome size (bp) of *M. bracteata* was then estimated by using the following formula (Doležel et al., 2003):

$$\text{Genome size (bp)} = (0.978 \times 10^9) \times \text{DNA content (pg)}$$

3.6 Transient expression of anti-toxoplasma IgG

3.6.1 *Agrobacterium* suspension preparation

Primary culture was setup by culturing 100 µL of *A. tumefaciens* harboring pTRAkcHcLcTg130 from the -80 ºC glycerol stock (Section 3.2.15) in 50 mL of YEB (Appendix C7) supplemented with appropriate antibiotic based on the strains of *A. tumefaciens* used (Appendix C7). The culture was incubated at 28 ºC in dark condition with agitation of 120 rpm for overnight. Secondary culture was setup by scaling-up the primary culture with an OD_{600} of 0.03 to 500 mL in YEB (Appendix C7) supplemented with appropriate antibiotic based on the strains of *A. tumefaciens* used (Appendix C7). The culture was incubated at 28 ºC in dark condition with agitation of 120 rpm until the OD_{600} reach more than 1.90. Afterward, the culture was pelleted by centrifugation at 7,000 rpm for 4 min at room temperature and resuspended in 1 L of infiltration buffer (Appendix C9). The OD_{600} was adjusted to about ~1.0 and the culture was statically incubated at 28ºC for at least 1 hour prior to infiltration (Section 3.6.2) (Section 3.6.3).

3.6.2 Syringe agroinfiltration of *Mucuna bracteata*

Five week-old *M. bracteata* plant with three trifoliate leaves (Figure 3.6A) (Section 3.3.2) (Section 3.3.5) were syringe infiltrated according to the method of Leuzinger et al. (2013). *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 suspended in infiltration buffer (Appendix C9) (Section 3.6.1) was injected into the intercellular space of the leaf with a syringe without a needle.

3.6.3 Vacuum-assisted agroinfiltration of *Mucuna bracteata*

Five week-old *M. bracteata* plant with three trifoliate leaves (Figure 3.6A) (Section 3.3.2) (Section 3.3.5) were vacuum infiltrated with 1 L of infiltration buffer (Appendix C9) containing *A. tumefaciens* strains GV3101, EHA105 or LBA4404 harboring pTRAkcHcLcTg130 (Section 3.6.1) for 30 min (Figure 3.6C). Vacuum time was recorded once the pressure reached -25 inches of Hg/-0.84 BAR (100× kPa) using the oilless diaphragm vacuum pump (GAST, USA). After agroinfiltration, the plants were transferred back into the growth room. Non-infiltrated plants were considered as negative control. The bottom (first true leaf from germination), intermediate (second true leaf from germination) and top (third true leaf from germination) trifoliate leaves (Figure 3.6B) were harvested at 2, 4 or 6 days post infiltration as described in Section 3.4.1.

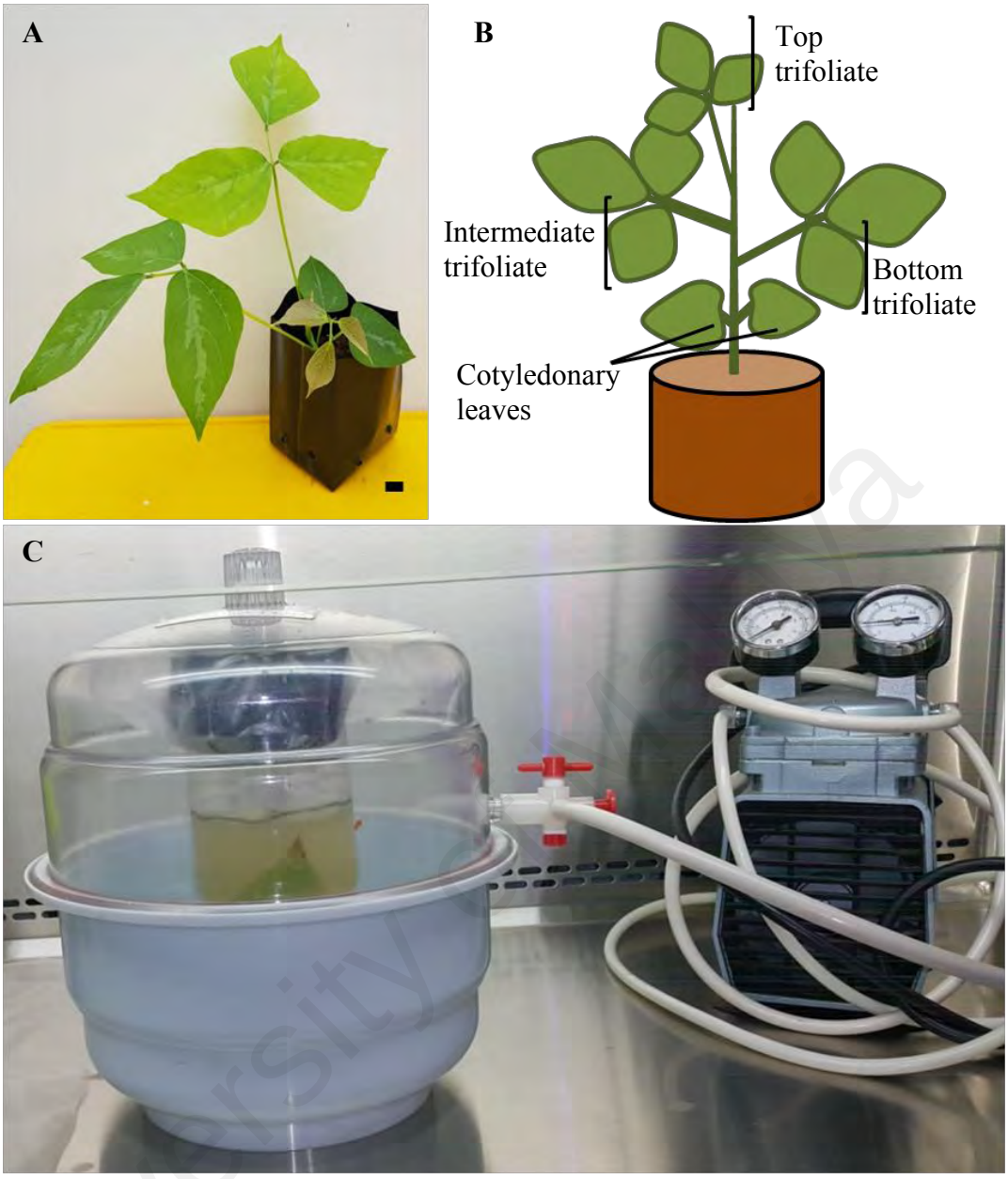


Figure 3.6: Setup for vacuum-assisted agroinfiltration of *Mucuna bracteata*. A: Five week-old *M. bracteata* plant with three trifoliate leaves was selected for infiltration (Bar = 1 cm). B: Trifoliate leaves position on a five week-old *M. bracteata* plant. C: Vacuum-assisted agroinfiltration of five week-old *M. bracteata* plant.

3.6.4 Syringe agroinfiltration of *Nicotiana benthamiana*

Seeds of *N. benthamiana* were sowed directly into polybags containing sterilized garden soil in a controlled environment (25-30 ^{o}C with 60 % humidity level) at Plant Biotech Facility, Universiti Malaya. The plants were watered twice a day.

The sixth leaf of 40 day-old *N. benthamiana* plant was syringe infiltrated according to the method of Leuzinger et al. (2013). *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 suspended in infiltration buffer (Appendix C9) (Section 3.6.1) was injected into the intercellular space of the leaf with a syringe without a needle until the entire leaf was infiltrated. Non-infiltrated plants were considered as negative control. The leaves were harvested after 6 days post infiltration as described in Section 3.4.1.

3.7 Stable expression of anti-toxoplasma IgG

3.7.1 *Agrobacterium* suspension preparation

Primary culture was setup by culturing 100 µL of *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 from the -80 ºC glycerol stock (Section 3.2.15) in 10 mL of YEB (Appendix C7) supplemented with carbenicillin (100 µg/mL) (Appendix C18) (Duchefa Biochemie, Netherlands), rifampicin (50 µg/mL) (Appendix C17) (Duchefa Biochemie, Netherlands) and G418 disulphate (50 µg/mL) (Appendix C19) (Duchefa Biochemie, Netherlands). The culture was incubated at 28 ºC in dark condition with agitation of 120 rpm for overnight. Secondary culture was setup by scaling-up the primary culture with an OD_{600} of 0.03 to 200 mL in YEB (Appendix C7) supplemented with carbenicillin (100 µg/mL) (Appendix C18) (Duchefa Biochemie, Netherlands), rifampicin (50 µg/mL) (Appendix C17) (Duchefa Biochemie, Netherlands) and G418 disulphate (50 µg/mL) (Appendix C19) (Duchefa Biochemie, Netherlands). The culture was incubated at 28 ºC in dark condition with agitation of 120 rpm until the OD_{600} reach more than ~1.90. Afterward, the culture was pelleted by centrifugation at 7,000 rpm for 4 minutes at room temperature and resuspended in 250 mL of infection buffer (Appendix C10). The OD_{600} was adjusted to about ~1.0 and the culture was statically incubated at 28ºC for at least 1 hour prior to use.

3.7.2 Natural regeneration capacity of *Mucuna bracteata* under antibiotic selection pressure

3.7.2.1 Shoot regeneration capacity

Cotyledonary node explants (Figure 3.4, right panel) that were prepared as described in Section 3.5.5 were cultured on MS media (Appendix D1) supplemented with 4.44 µM of BAP (Appendix D10) (Duchefa Biochemie, Netherlands) and various concentration of kanamycin (50-150 µg/mL) (Appendix C21) (Duchefa Biochemie, Netherlands) or G418 disulphate (12.5-75 µg/mL) (Appendix C19) (Duchefa Biochemie, Netherlands). All cultures were maintained in growing condition as described in Section 3.3.4. The shoot height and number of shoots per explants were recorded after one month of culture.

3.7.2.2 Root regeneration capacity

Elongated shoots (5-20 cm in length) were excised from shoot clumps (Figure 3.5) (Section 3.5.5) and transferred to MS media (Appendix D1) supplemented with 10.7 µM of NAA (Appendix D11) (Duchefa Biochemie, Netherlands) and various concentration of G418 disulphate (12.5-75 µg/mL) (Appendix C19) (Duchefa Biochemie, Netherlands). All cultures were maintained in growing condition as described in Section 3.3.4. The percentage of shoots with root formation, number of root per shoot and root length were recorded after one month of culture.

3.7.3 Cotyledonary node transformation of *Mucuna bracteata*

3.7.3.1 Explants preparation

Cotyledonary node explants (Figure 3.4: right panel) that were prepared as described in Section 3.5.5 were immersed in 10 mM ascorbic acid solution (Appendix C11). The explants were rinsed twice in sdH_2O to get rid of excessive leached phenolic compounds prior to infection (Section 3.7.3.2).

3.7.3.2 Explants infection

Explants (Section 3.7.3.1) were infected by immersing in *Agrobacterium* suspension (Section 3.7.1) for 10, 20 or 30 min at 28 ^{o}C in dark condition with agitation of 120 rpm.

3.7.3.3 Explants co-cultivation

Infected explants (Section 3.7.3.2) were blotted on sterile paper towel to remove excess *Agrobacterium* culture and air-dried for 40 min. The explants were co-cultured on a single layer of 70 mm filter paper (Whatman, UK) moistened with 1 mL of infection buffer (Appendix C10) for 2, 4 or 6 days in dark condition at 28 ^{o}C. After co-cultivation period, the explants were washed at least five times with vigorous stirring in ample sdH_2O, blotted dry on sterile paper towel and air-dried for 30 min.

3.7.3.4 Maintenance and regeneration of putative transformed explants

Co-cultivated explants (Section 3.7.3.3) were cultured on MB selection media (Appendix D6). All cultures were maintained in growing condition as described in Section 3.3.4.

3.7.3.5 Analysis of putative transformed explants

Genomic DNA was extracted (Section 3.2.2) from tissue sample (leaf or shoot tip) obtained from shoots generated on MB selection media (Appendix D6) after one month of culture (Section 3.7.3.4). The extracted genomic DNA was used as DNA template for amplification (Section 3.2.6) using CBT03 primer (Appendix B: Table 2) and TRAnpt primer (Appendix B: Table 1). The PCR products were analyzed as described in Section 3.2.7.

For PCR analysis using CBT03 primer, genomic DNA of non-transgenic *M. bracteata* extracted using either DNeasy Plant Mini Kit (QIAGEN, USA) (Section 3.2.1) or Edward method (Section 3.2.2) were used as DNA template for positive controls. For the negative control, sdH_2O was used as DNA template.

For PCR analysis using TRAnpt primer, sdH_2O and genomic DNA of non-transgenic *M. bracteata* extracted using DNeasy Plant Mini Kit (QIAGEN, USA) (Section 3.2.1) were used as DNA template for negative controls. For the positive control, plasmid pTRAkcHcLcTg130 was used as DNA template.

3.7.4 Leaf discs transformation of *Nicotiana tabacum* cv. SR1

3.7.4.1 Explants preparation

Leaf discs explants were prepared by excising leaves of 1 month old *N. tabacum* cv. SR1 (Section 3.3.3) into ~1cm × 1cm. The explants were cultured on TSM media (Appendix D7) for two days in growing condition as described in Section 3.3.4.

3.7.4.2 Explants infection

The explants (Section 3.7.4.1) were infected by immersing in *Agrobacterium* suspension (Section 3.7.1) for 10 min at 28 ^{o}C in dark condition with agitation of 120 rpm.

3.7.4.3 Explants co-cultivation

Infected explants (Section 3.7.4.2) were blotted on sterile paper towel to remove excess *Agrobacterium* culture and air-dried for 40 min. The explants were co-cultured on TSM media (Appendix D7) for three days in dark condition at 25 ^{o}C. After co-cultivation period, the explants were washed at least five times with vigorous stirring in ample sdH_2O, blotted dry on sterile paper towel and air-dried for 30 min.

3.7.4.4 Maintenance and regeneration of putative transformed explants

Co-cultivated explants (Section 3.7.4.3) were cultured on NT selection media (Appendix D8) and maintained in growing condition as described in Section 3.3.4 for one month.

3.7.4.5 Analysis of putative transformed explants

Genomic DNA was extracted (Section 3.2.2) from the leaves of shoots generated on NT selection media (Appendix D8) after one month of culture (Section 3.7.4.4). The extracted genomic DNA was used as DNA template for amplification (Section 3.2.4) using TRAnpt primer (Appendix B: Table 1). The PCR products were analyzed as described in Section 3.2.7. Shoot with transgene presence was considered as transgenic T_0 shoot.

3.7.4.6 Root induction

Transgenic T_0 shoot (Section 3.7.4.5) was transferred onto TRM media (Appendix D9) and maintained in growing condition as described in Section 3.3.4 for one month. Rooted transgenic T_0 shoot was acclimatized as described in Section 3.3.5.

3.7.4.7 Self-crossing of T_0 plants

Acclimatized transgenic T_0 plants (Section 3.7.4.6) was maintained in Plant Biotech Facility, Universiti Malaya (Section 3.3.5) until flowering. Once the flower bud started to bloom, the anther was brushed against its own stigma to pollinate itself. The flower head was then covered with a light-weight paper bag to prevent cross pollination from other flowers until the formation of seed pod. Seeds from dried seed pod were collected and designated as T_1 generation.

3.7.4.8 T_1 germination

T_1 seeds were germinated (Section 3.3.3) on NT selection media (Appendix D8) with and without the supplementation of kanamycin (Appendix C21) (Duchefa Biochemie, Netherlands) in growing condition as described in Section 3.3.4. The number of seeds germinated was recorded. Two-week old seedlings were randomly selected to be acclimatized as described in Section 3.3.5.

3.7.4.9 Phenotypic assessment, transgene presence verification and anti-toxoplasma IgG expression analysis

When the acclimatized T_1 plants (Section 3.7.4.8) were 40 day-old, the number of leaves and the stem height of each plant were recorded whereas the sixth leaf of each plant was harvested as described in Section 3.4.1. Genomic DNA was extracted (Section 3.2.1) as DNA template for transgene presence verification (Section 3.2.4) using TRAnpt primer (Appendix B: Table 1). The PCR products were analyzed as described in Section 3.2.7. Anti-toxoplasma IgG expression was analyzed as described from Section 3.4.2 until Section 3.4.8.

3.7.4.10 Self-crossing of T_1 plants

Acclimatized transgenic T_1 plants (Section 3.7.4.8) were maintained in Plant Biotech Facility, Universiti Malaya (Section 3.3.5) until flowering. The number of days the plants took to flower was recorded. Self-pollination was carried out as described in Section 3.7.4.7. Seeds from the dried seed pods were collected, designated as T_2 generation and kept for inventory.

3.8 Data collection and statistical analysis

For Section 3.5.1 – Section 3.5.6 and Section 3.7.2 – Section 3.7.4, each treatment consisted of 30 explants and the entire experiment was repeated three times.

For Section 3.6.3 and Section 3.6.4, each treatment consisted of three plants and the entire experiment was repeated three times.

Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Duncan's multiple-range test at a significance level of $p < 0.05$. Experiments that involved more than one classification criterion were analyzed with two-way analysis of variance.

CHAPTER 4: RESULTS

4.1 pTRAkcHcLcTg130 plant expression vector construction

4.1.1 Establishment of fresh glycerol stocks of *E. coli* TOP10 harboring either pTRAkc-Hc or pTRAkc-Lc

Glycerol stocks of *E. coli* TOP10 harboring pTRAkc-Hc and pTRAkc-Lc (Section 3.1) were streaked on LB selection plate (Appendix C6). Colonies formed on the LB selection plate (Appendix C6) were picked for PCR colony analysis using Hc primer (Appendix B: Table 1) for pTRAkc-Hc and Lc primer (Appendix B: Table 1) for pTRAkc-Lc as described in Section 3.2.14. The results are presented in Figure 4.1.

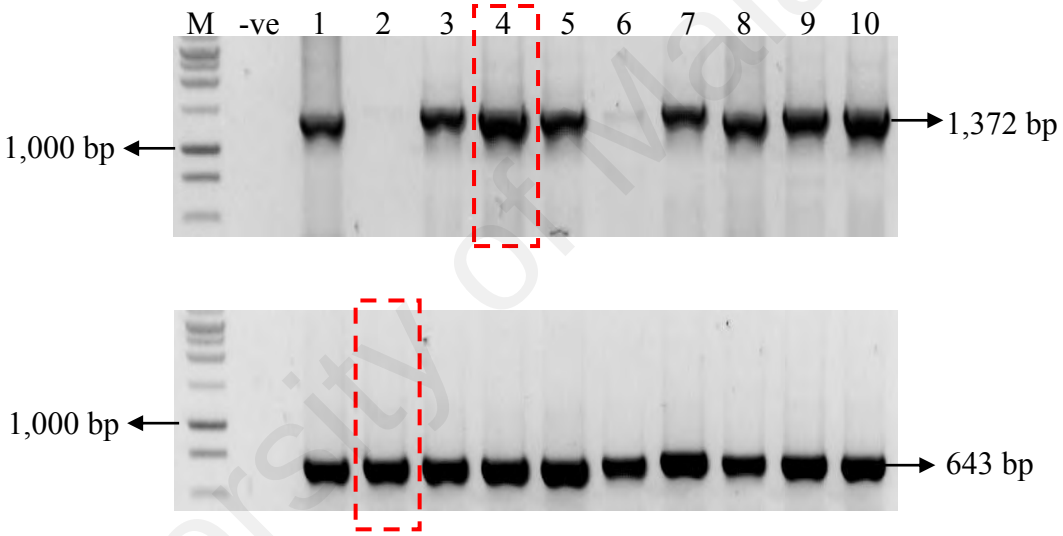


Figure 4.1: Glycerol stock PCR colony analysis using Hc primer for pTRAkc-Hc (top panel) and Lc primer for pTRAkc-Lc (bottom panel). Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane –ve: PCR negative control using sdH2O as DNA template; Lanes 1-10: selected colonies.

Nine colonies from *E. coli* TOP10 harboring pTRAkc-Hc yielded a band with the expected size of 1,372 bp (Figure 4.1: top panel) whereas all ten colonies from *E. coli* TOP10 harboring pTRAkc-Lc yield a band with the expected size of 643 bp (Figure 4.1: bottom panel). The fourth colony of *E. coli* TOP10 harboring pTRAkc-Hc and the second colony of *E. coli* TOP10 harboring pTRAkc-Lc were chosen to establish an overnight suspension culture (Section 3.2.16) followed with bacterial glycerol stock (Section 3.2.15). The prepared glycerol stocks were used in subsequent experiments.

4.1.2 Introduction of pTRAkc-rfp-ERH into NEB® 5-alpha F' I^q *E. coli*

The plasmid pTRAkc-rfp-ERH (Section 3.1) was introduced into NEB® 5-alpha F' I^q *E. coli* (New England Biolabs, USA) as described in Section 3.2.11. Colonies formed on the LB selection plate (Appendix C6) were picked for PCR colony analysis using InPra2 primer (Appendix B: Table 1) as described in Section 3.2.14. The results are presented in Figure 4.2.

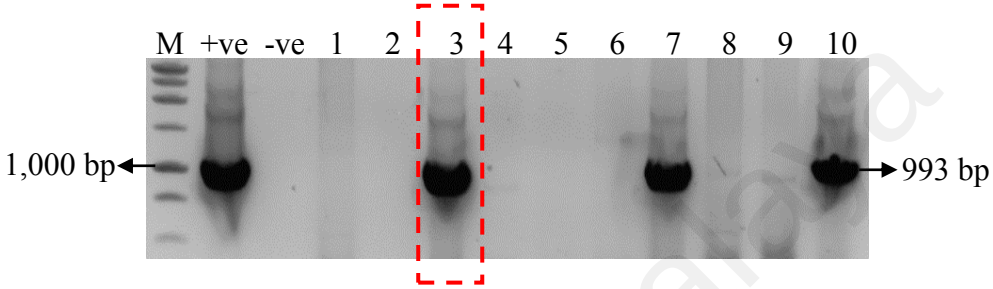


Figure 4.2: PCR colony analysis of NEB® 5-alpha F' I^q *E. coli* (New England Biolabs, USA) transformation with pTRAkc-rfp-ERH using InPra2 primer. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane +ve: PCR positive control using pTRAkc-rfp-ERH as DNA template; Lane –ve: PCR negative control using sdH_2O as DNA template; Lanes 1-10: selected colonies.

PCR colony analysis showed that three colonies that were selected from NEB® 5-alpha F' I^q *E. coli* transformation with pTRAkc-rfp-ERH yield a band with the expected size of 993 bp (Figure 4.2). The third colony of NEB® 5-alpha F' I^q *E. coli* harboring pTRAkc-rfp-ERH was chosen to establish overnight suspension culture (Section 3.2.16) followed with bacterial glycerol stock (Section 3.2.15).

4.1.3 Reconstruction of pTRAkc-Hc and pTRAkc-Lc into pTRAkcHcTg130 and pTRAkcLcTg130, respectively

Compared to plasmid pTRAkc-rfp-ERH (Figure 3.3), the plasmids pTRAkc-Hc (Figure 3.1) and pTRAkc-Lc (Figure 3.2) did not contain a selectable marker that can be used for stable transformation (Section 3.1). The plasmid pTRAkc-rfp-ERH contains an *nptII* gene that confers resistance towards kanamycin and G418 disulphate (Section 3.1) (Figure 3.3). Thus, the heavy chain fragment of the anti-toxoplasma IgG within pTRAkc-Hc and the light chain fragment of anti-toxoplasma IgG within pTRAkc-Lc were excised from their vector backbone and ligated into the rfp region of pTRAkc-rfp-ERH to construct pTRAkcHcTg130 and pTRAkcLcTg130, respectively.

Plasmids pTRAkc-rfp-ERH, pTRAkc-Hc and pTRAkc-Lc were extracted from overnight cultures (Section 3.2.3) and digested as described in Section 3.2.8 with FastDigest EcoRI (Thermo Scientific, USA) and FastDigest BamHI (Thermo Scientific, USA). Restriction digests were analyzed via gel electrophoresis (Section 3.2.7) and presented in Figure 4.3.

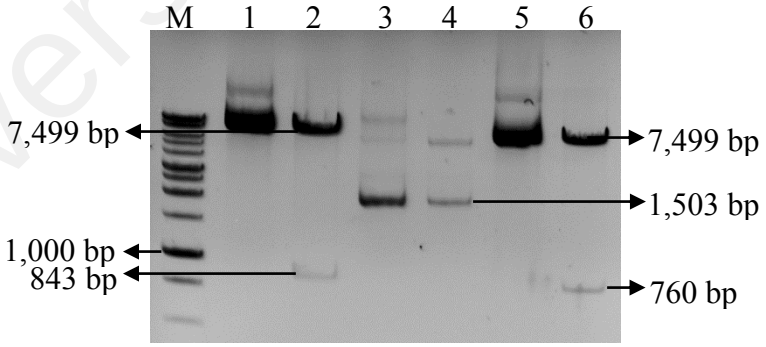


Figure 4.3: Restriction digests of plasmids with FastDigest EcoRI (Thermo Scientific, USA) and FastDigest BamHI (Thermo Scientific, USA). Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane 1: Uncut pTRAkc-rfp-ERH; Lane 2: Digested pTRAkc-rfp-ERH; Lane 3: Uncut pTRAkc-Hc; Lane 4: Digested pTRAkc-Hc; Lane 5: Uncut pTRAkc-Lc; Lane 6: Digested pTRAkc-Lc.

Digestion of all plasmids produced two bands of expected size. The larger fragment with the expected size of 7,499 bp from pTRAkc-rfp-ERH digestion (Figure 4.3: Lane 2) was the vector backbone devoid of rfp region (843 bp). The smaller fragments with the expected size of 1,503 bp from pTRAkc-Hc digestion (Figure 4.3: Lane 4) and 760 bp from pTRAkc-Lc (Figure 4.3: Lane 6) digestion contained the heavy chain and the light chain fragments of anti-toxoplasma IgG, respectively. The larger fragments with the expected size of 7,499 bp from both pTRAkc-Hc digestion (Figure 4.3: Lane 4) and pTRAkc-Lc digestion (Figure 4.3: Lane 6) were the vector backbone devoid of the antibody fragment region All fragments of interest (7,499 bp from pTRAkc-rfp-ERH digestion; 1,503 bp from pTRAkc-Hc digestion; 760 bp from pTRAkc-Lc digestion) were gel extracted and purified as described in Section 3.2.9.

The purified heavy chain and light chain fragments were then ligated separately into the pTRAkc-rfp-ERH vector backbone (Section 3.2.10) to form pTRAkcHcTg130 (Figure 4.4) and pTRAkcLcTg130 (Figure 4.5), respectively.

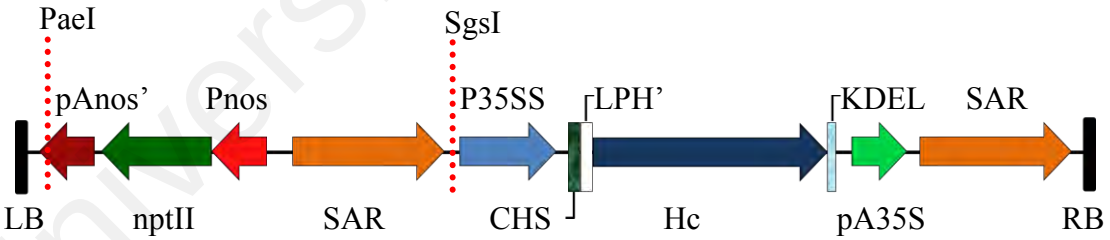


Figure 4.4: Schematic view of pTRAkcHcTg130. LB, left border; PaeI, PaeI restriction site; pAnos', nopaline synthase gene polyadenylation signal; nptII, neomycin phosphotransferase II; Pnos, nopaline synthase promoter; SAR, scaffold attachment region of the tobacco RB7 gene (GenBank U67919); SgsI, SgsI restriction site; P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; CHS, chalcone synthase 5'-untranslated region; LPH, codon-optimized murine signal peptide of mAB24; Hc, heavy chain sequence of anti-toxoplasma IgG; KDEL, endoplasmic reticulum retention signal; pA35S, CaMV 35S polyadenylation signal; RB, right border.

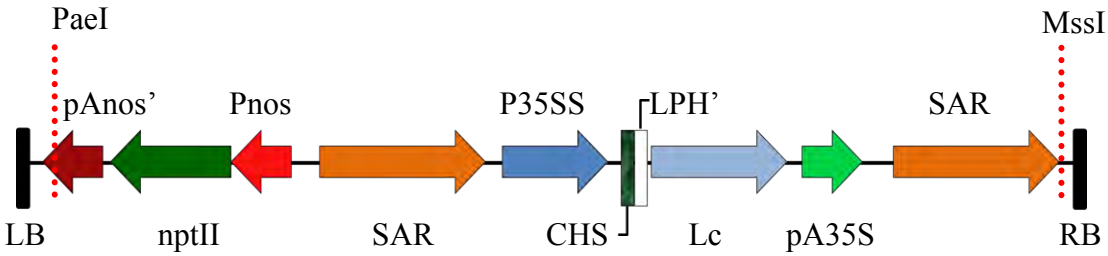


Figure 4.5: Schematic view of pTRAkcLcTg130. LB: left border; PaeI, PaeI restriction site; pAnos', nopaline synthase gene polyadenylation signal; nptII: neomycin phosphotransferase II; Pnos: nopaline synthase promoter; SAR: scaffold attachment region of the tobacco RB7 gene (GenBank U67919); P35SS: CaMV 35S promoter with duplicated transcriptional enhancer; CHS: chalcone synthase 5'-untranslated region; LPH': codon-optimized murine signal peptide of mAB24; Lc: light chain sequence of anti-toxoplasma IgG; pA35S: CaMV 35S polyadenylation signal; MssI, MssI restriction site; RB: right border.

The ligated products were then introduced into NEB® 5-alpha F' I^q *E. coli* (New England Biolabs, USA) as described in Section 3.2.11. Colonies formed on the LB selection plate (Appendix C6) were picked for PCR colony analysis using InPra2 primer (Appendix B: Table 1) as described in Section 3.2.14. The results are presented in Figure 4.6.

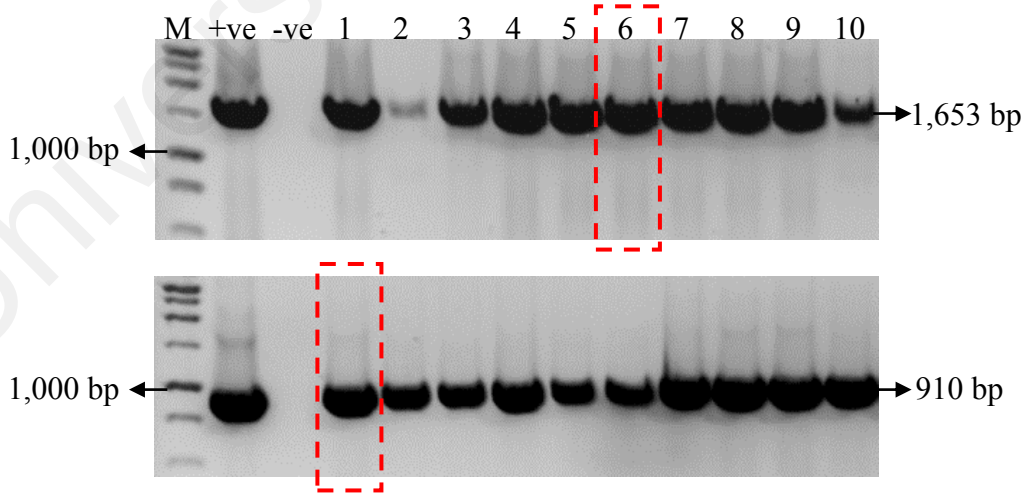


Figure 4.6: PCR colony analysis using InPra2 primer. NEB® 5-alpha F' I^q *E. coli* (New England Biolabs, USA) was introduced with pTRAkcHcTg130 (top panel) and pTRAkcLcTg130 (bottom panel). Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane +ve: PCR positive control (pTRAkc-Hc and pTRAkc-Lc as DNA template for top and bottom panel, respectively); Lane –ve: PCR negative control using sdH_2O as DNA template; Lanes 1-10: selected colonies.

PCR colony analysis showed that all selected colonies yielded a band with the expected size of 1,653 bp (Figure 4.6: top panel) from NEB® 5-alpha F' I^q *E. coli* transformation with pTRAkcHcTg130 and 910 bp (Figure 4.6: bottom panel) from NEB® 5-alpha F' I^q *E. coli* transformation with pTRAkcLcTg130. The sixth colony from NEB® 5-alpha F' I^q *E. coli* transformation with pTRAkcHcTg130 and the first colony from NEB® 5-alpha F' I^q *E. coli* transformation with pTRAkcLcTg130 were chosen to establish overnight suspension culture (Section 3.2.15) followed with bacterial glycerol stock (Section 3.2.14).

4.1.4 Generation of tandem heavy chain and light chain expression plasmid, pTRAkcHcLcTg130

Plasmids pTRAkcHcTg130 and pTRAkcLcTg130 were extracted from overnight cultures (Section 3.2.3) and digested as described in Section 3.2.8. Plasmid pTRAkcHcTg130 was digested with FastDigest *SgsI* (Thermo Scientific, USA) and FastDigest *PaeI* (Thermo Scientific, USA) whereas plasmid pTRAkcLcTg130 was digested with FastDigest *PaeI* (Thermo Scientific, USA) and FastDigest *MssI* (Thermo Scientific, USA). Restriction digests were analyzed via gel electrophoresis (Section 3.2.7) and the results are presented in Figure 4.7.

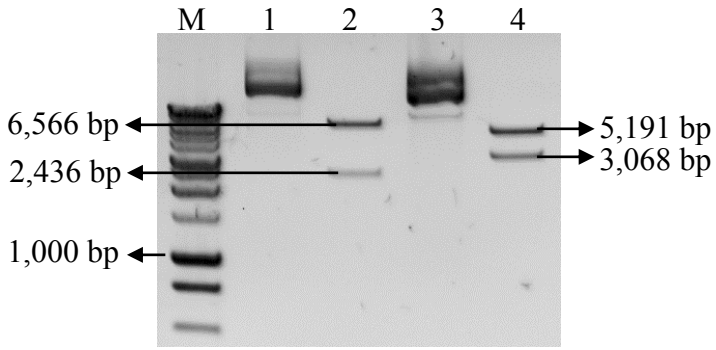


Figure 4.7: Restriction digests of plasmids with restriction enzymes. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane 1: Uncut pTRAkcHcTg130; Lane 2: pTRAkcHcTg130 digested with FastDigest *SgsI* (Thermo Scientific, USA) and FastDigest *PaeI* (Thermo Scientific, USA); Lane 3: Uncut pTRAkcLcTg130; Lane 4: pTRAkcLcTg130 digested with FastDigest *PaeI* (Thermo Scientific, USA) and FastDigest *MssI* (Thermo Scientific, USA).

Digestion of pTRAkcHcTg130 produced two fragments of 6,566 bp and 2,436 bp (Figure 4.7: Lane 2) whereas digestion of pTRAkcLcTg130 produced two fragments of 5,191 bp and 3,068 bp (Figure 4.7: Lane 4). The larger fragments from both restriction digests were gel extracted, purified (Section 3.2.9) and ligated (Section 3.2.10) to generate a tandem heavy chain and light chain expression plasmid, pTRAkcHcLcTg130 (Figure 4.8).

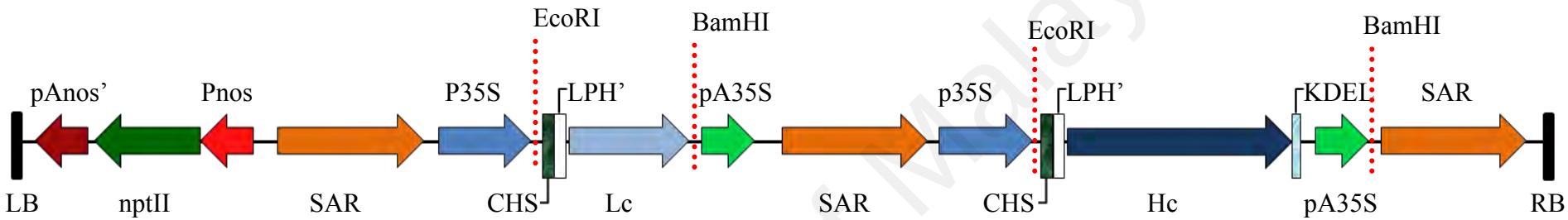


Figure 4.8: Schematic view of pTRAkcHcLcTg130. LB, left border; pAnos': nopaline synthase gene polyadenylation signal; nptII, neomycin phosphotransferase II; Pnos, nopaline synthase promoter; SAR, scaffold attachment region of the tobacco RB7 gene (GenBank U67919); P35SS, CaMV 35S promoter with duplicated transcriptional enhancer; EcoRI, EcoRI restriction site; CHS, chalcone synthase 5'-untranslated region; LPH, codon-optimized murine signal peptide of mAB24; Lc, light chain sequence of anti-toxoplasma IgG; BamHI, BamHI restriction site; pA35S, CaMV 35S polyadenylation signal; Hc, heavy chain sequence of anti-toxoplasma IgG; KDEL, endoplasmic reticulum retention signal; RB, right border.

The disappearance of fragments with lower molecular weight and the appearance of fragment with higher molecular weight in the ligation mixture indicated a successful ligation (Figure 4.9: Lane 2).

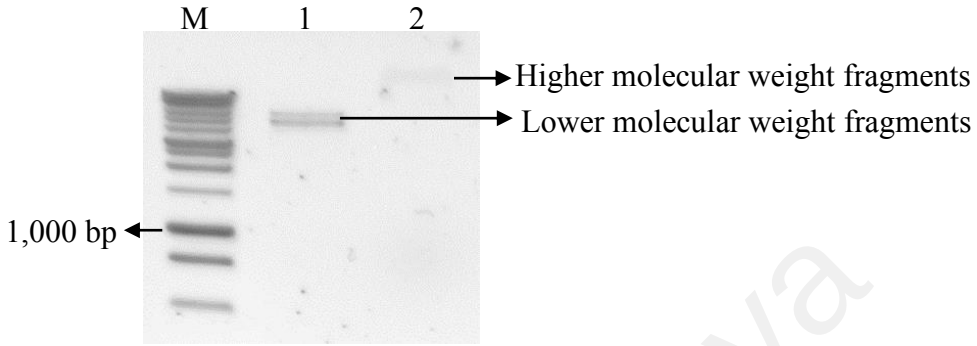


Figure 4.9: Ligation reaction of selected fragments from pTRAkcHcTg130 and pTRAkcLcTg130 digestion reaction. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane 1: Unligated reaction of selected fragments from pTRAkcHcTg130 and pTRAkcLcTg130 digestion reaction; Lane 2: Ligation reaction of selected fragments from pTRAkcHcTg130 and pTRAkcLcTg130 digestion reaction.

The ligated products were then introduced into NEB® 5-alpha F' I^q *E. coli* (New England Biolabs, USA) as described in Section 3.2.11. Colonies formed on the LB selection plate (Appendix C6) were picked for PCR colony analysis using InPra2 primer (Appendix B: Table 1) as described in Section 3.2.14. The results are presented in Figure 4.10.

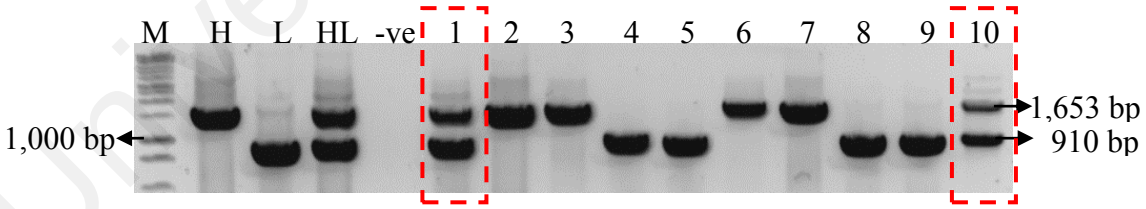


Figure 4.10: PCR colony analysis using InPra2 primer. NEB® 5-alpha F' I^q *E. coli* (New England Biolabs, USA) was introduced with pTRAkcHcLcTg130. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane H: PCR positive control using pTRAkcHcTg130 as DNA template; Lane L: PCR positive control using pTRAkcLcTg130 as DNA template; Lane HL: PCR positive control using mixture of pTRAkHcTg130 and pTRAkcLcTg130 as DNA template; Lane –ve: PCR negative control using sdH2O as DNA template; Lanes 1-10: selected colonies.

Out of the ten colonies that were selected for PCR colony analysis, only two colonies (Figure 4.10: Lane 1 and Lane 10) showed the presence of both heavy chain (1,653 bp) and light chain (910 bp) fragments of anti-toxoplasma IgG. These two colonies were chosen to establish overnight suspension culture (Section 3.2.16) followed by plasmid extraction (Section 3.2.3) and diagnostic restriction digestion (Section 3.2.8) for pTRAkcHcLcTg130 verification. The plasmid was digested with either FastDigest *EcoRI* (Thermo Scientific, USA) only or with combination of FastDigest *BamHI* (Thermo Scientific, USA). Restriction digests were analyzed via gel electrophoresis (Section 3.2.7) and presented in Figure 4.11.

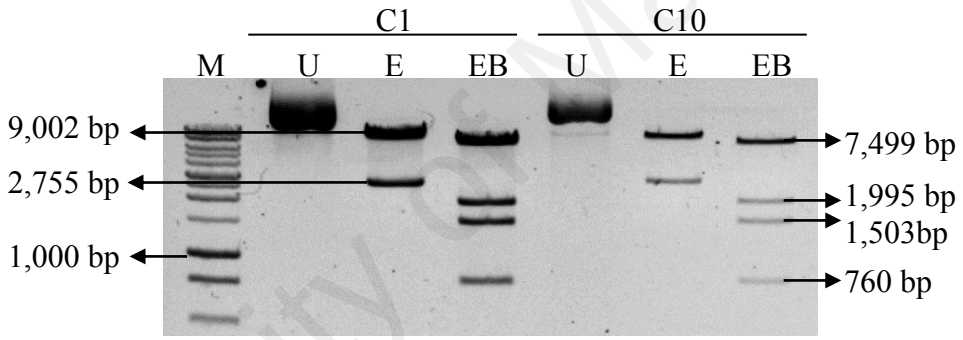


Figure 4.11: Diagnostic restriction digests of pTRAkcHcLcTg130 with FastDigest *EcoRI* (Thermo Scientific, USA) and FastDigest *BamHI* (Thermo Scientific, USA). Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); C1: The first colony from NEB® 5-alpha F' I^q *E. coli* (New England Biolabs, USA) transformation with pTRAkcHcLcTg130; C10: The tenth colony from NEB® 5-alpha F' I^q *E. coli* (New England Biolabs, USA) transformation with pTRAkcHcLcTg130; U: undigested pTRAkcHcLcTg130; E: pTRAkcHcLcTg130 digested with FastDigest EcoRI (Thermo Scientific, USA); EB: pTRAkcHcLcTg130 digested with FastDigest EcoRI (Thermo Scientific, USA) and FastDigest BamHI (Thermo Scientific, USA).

Digestion of plasmid from both colonies showed similar bands pattern (Figure 4.11). Single digestion with FastDigest EcoRI (Thermo Scientific, USA) produced two bands with the expected size of 9,002 bp and 2,755 bp (Figure 4.11, Lane E). Double digestion with FastDigest EcoRI (Thermo Scientific, USA) and FastDigest BamHI (Thermo Scientific, USA) produced four bands with expected sizes of 7,499 bp, 1,995 bp, 1,503 bp and 760 bp (Figure 4.11, Lane EB).

Plasmid was extracted from suspension culture of the first colony (Section 3.2.3) and used as DNA template to amplify the heavy chain and the light chain fragments (Section 3.2.6). The fragments were gel extracted and purified (Section 3.2.9) for sequencing (Section 3.2.17). Since sequencing would normally yield good quality of chromatogram only for the first 1,000 bp, the heavy chain fragment was sequenced using two sets of primers, each amplifying different segment of the heavy chain fragment. The list of the primers used for sequencing the heavy chain and light chain fragments were listed in Appendix B: Table 3. The sequencing result showed that there was no mutation in the sequences of both the heavy (Appendix F) and the light chain (Appendix G) fragments of anti-toxoplasma IgG. Thus, the colony was chosen to establish glycerol stock for usage in subsequent experiments of the study.

4.2 Introduction of pTRAkcHcLcTg130 into *Agrobacterium tumefaciens*

The plasmid pTRAkcHcLcTg130 was introduced into competent *A. tumefaciens* strains GV3101, EHA105 and LBA4404 (Section 3.2.12-Section 3.2.13). Colonies grown on selection plate were selected for PCR colony analysis (Section 3.2.14). The results are presented in Figure 4.12.

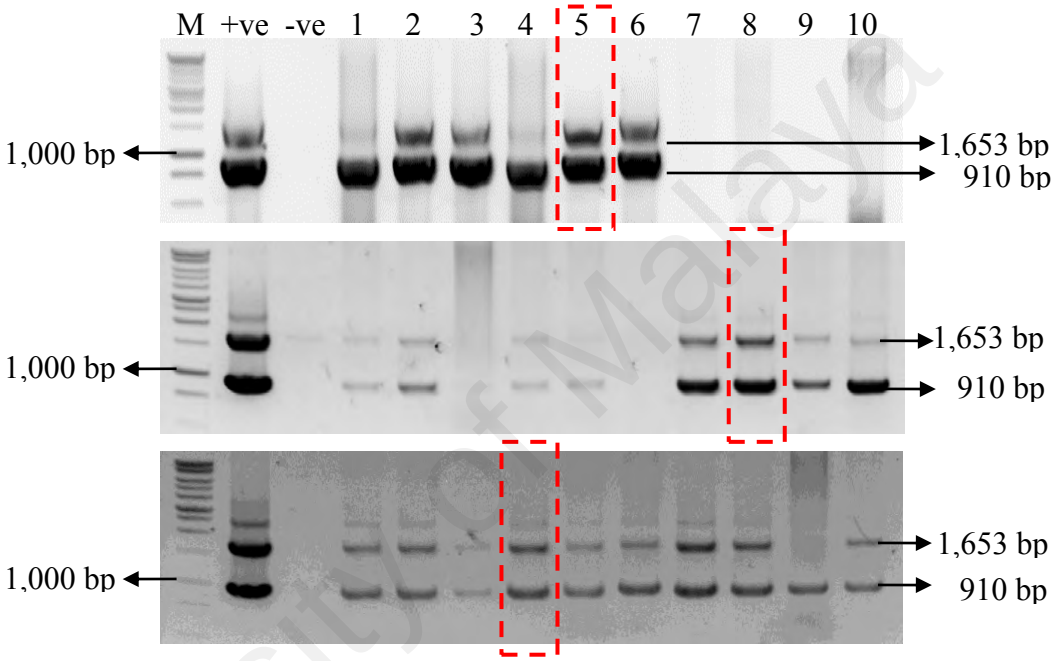


Figure 4.12: PCR colony analysis using InPra2 primer. The plasmid pTRAkcHcLcTg130 was introduced into competent cells of *A. tumefaciens* strain GV3101 (top panel), EHA 105 (middle panel) and LBA4404 (bottom panel). Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane +ve: PCR positive control using pTRAkcHcLcTg130 as DNA template; Lane –ve: PCR negative control using sdH2O as DNA template; Lanes 1-10: selected colonies.

The expected band sizes were 1,653 bp for the heavy chain sequence and 910 bp for the light chain sequence. PCR colony analysis showed that not all colonies contained both of these sequences. The fifth colony from *A. tumefaciens* strain GV3101 transformation with pTRAkcHcLcTg130 (Figure 4.12: top panel), the eighth colony from *A. tumefaciens* strain EHA105 transformation with pTRAkcHcLcTg130 (Figure 4.12: middle panel) and the fourth colony from *A. tumefaciens* strain LBA4404 transformation with pTRAkcHcLcTg130 (Figure 4.12: bottom panel) were chosen to establish an overnight suspension culture (Section 3.2.16) followed with bacterial glycerol stock (Section 3.2.15) for use in subsequent experiments of the study.

4.3 Standard curve for Bradford assay

4.3.1 Using PBS buffer

A standard curve was constructed using bovine serum albumin (BSA) (VWR Life Science, USA) dissolved in PBS (Amresco, USA) as described in Section 3.4.3.

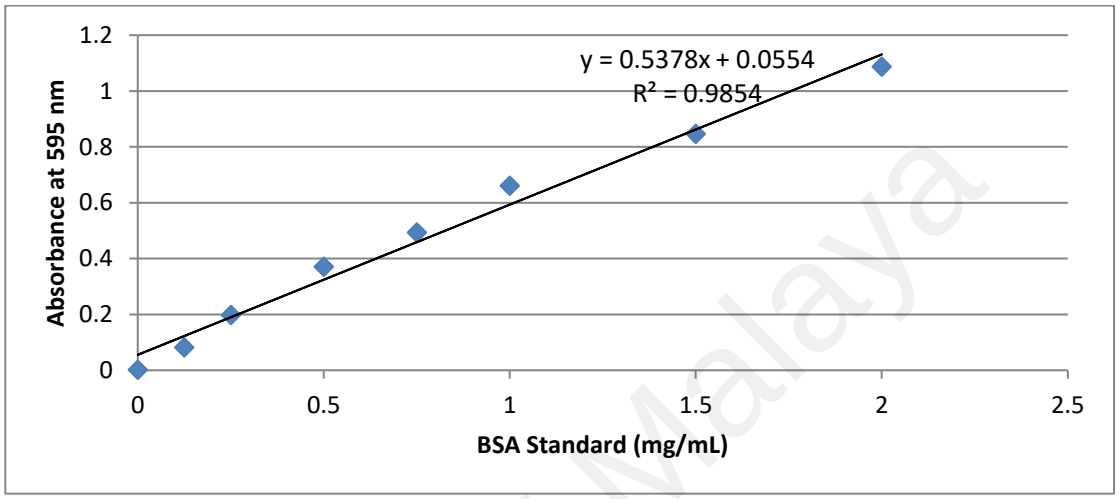


Figure 4.13: Standard curve for Bradford assay constructed using bovine serum albumin (BSA) (VWR Life Science, USA) dissolved in PBS (Amresco, USA).

A linear regression with an R^2 value of 0.9854 fell within the acceptable range. Thus, the generated standard curve was used to calculate protein concentration in subsequent experiments where crude protein was extracted using PBS (Amresco, USA).

4.3.2 Using NEB buffer

A standard curve was constructed using bovine serum albumin (BSA) (VWR Life Science, USA) dissolved in NEB (Appendix E4) as described in Section 3.4.3.

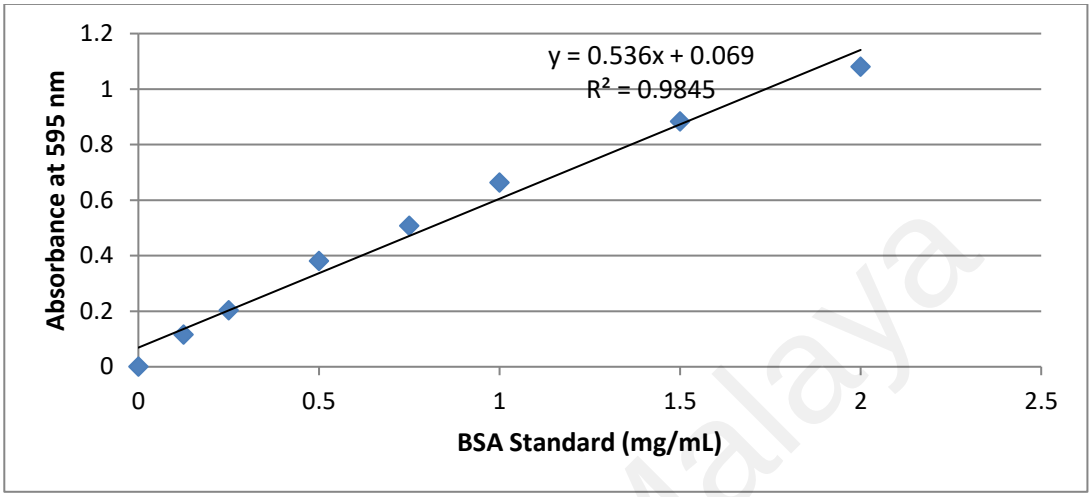


Figure 4.14: Standard curve for Bradford assay constructed using bovine serum albumin (BSA) (VWR Life Science, USA) dissolved in NEB (Appendix E4).

A linear regression with an R^2 value of 0.9845 fell within the acceptable range. Thus, the generated standard curve was used to calculate protein concentration in subsequent experiments where crude protein was extracted using NEB (Appendix E4).

4.4 *In vitro* propagation of *Mucuna bracteata*

4.4.1 Seeds scarification

Seeds of *M. bracteata* were encapsulated with hard and thick testa layer which made them impermeable to water and air. The process of weakening this testa layer to allow water and air permeability is known as seeds scarification. In this study, seeds of *M. bracteata* were scarified using concentrated sulphuric acid (98%) (Section 3.3.2) (Section 3.5.1). Compared to the shiny and smooth surfaces of non-scarified seeds, scarified seeds displayed rough surfaces (Figure 4.15).

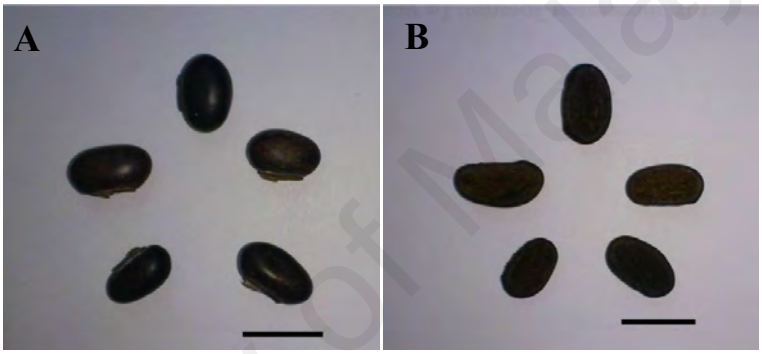


Figure 4.15: Seeds of *Mucuna bracteata*. A: *M. bracteata* seeds enclosed with a testa layer. B: Scarified *M. bracteata* seeds without the testa layer. Bar = 1 cm.

The seeds were submerged in concentrated sulphuric acid (98%) for 10, 30, and 60 min to determine the suitable exposure time. The study showed that seeds submerged in sulphuric acid for 30 min have the highest percentage of germinated seeds (36.4% at Day 12) compared to the other exposure times (Table 4.1).

Table 4.1: The percentage of seed germination of *Mucuna bracteata* under different exposure times in sulfuric acid

Exposure period of sulfuric acid (min)	Percentage of seed germination (%)		
	Day 4	Day 8	Day 12
0	$0.0 \pm 0.0_{a}$	$0.0 \pm 0.0_{a}$	$0.0 \pm 0.0_{a}$
10	$6.9 \pm 2.82_{ab}$	$10.3 \pm 4.10_{ab}$	$11.1 \pm 4.80_{ab}$
30	$11.4 \pm 4.31_{b}$	$30.8 \pm 5.10_{c}$	$36.4 \pm 6.30_{c}$
60	$12.2 \pm 1.11_{b}$	$21.1 \pm 4.84_{bc}$	$22.2 \pm 4.01_{bc}$

*Mean ± standard error values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p<0.05$

4.4.2 Seeds surface sterilization

M. bracteata seeds that have been scarified for 30 min in sulfuric acid were surface sterilized using three types of treatments (95% ethanol, 50% Clorox® or a combination of 95% ethanol and 50% Clorox®) (Section 3.3.2) (Section 3.5.2). We found that the percentage of contaminated seeds treated in 95% ethanol and 50% Clorox® alone was 6% and 7.3%, respectively, compared to the non-treated seeds (2.4%) (Table 4.2). Although the combination of ethanol and Clorox® reduced the percentage of contaminated seeds to 0.7%, differences between the treatments were not significant. In addition, different treatments did not affect the percentage of seed germination.

Table 4.2: The percentage of *Mucuna bracteata* seed germination and contamination using different sterilization methods

Treatment	Percentage of seed germination (%)			Contamination (%)
	Day 4	Day 8	Day 12	
No treatment	$1.2 \pm 1.37_a$	$23.0 \pm 3.91_a$	$33.1 \pm 3.23_a$	$2.4 \pm 1.21_a$
95% EtOH	$1.3 \pm 0.99_a$	$19.2 \pm 3.09_a$	$32.7 \pm 3.69_a$	$6.0 \pm 6.00_a$
50% Clorox®	$6.0 \pm 2.39_a$	$23.3 \pm 3.85_a$	$34.7 \pm 4.81_a$	$7.3 \pm 6.36_a$
95% EtOH: 50% Clorox®	$2.4 \pm 1.54_a$	$13.3 \pm 3.39_a$	$28.3 \pm 4.70_a$	$0.7 \pm 0.67_a$

*Mean ± standard error values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p<0.05$

4.4.3 Seeds priming

Two types of priming were evaluated in this study; hydropriming and osmopriming (Section 3.5.3) (Section 3.3.2).

In hydropriming, *M. bracteata* seeds were imbibed in sdH_2O for 0, 6, 24, and 48 h and cultured on double layers of wet cotton roll with different volumes of sdH_2O to promote seed germination. The study showed that seeds imbibed for 6 h and cultured on wet cotton roll with 10 mL of sdH_2O produced the highest percentage of germinated seeds (43.0%) on the twelfth day (Table 4.3). Increased volumes of sdH_2O to the cotton roll and longer hydropriming period have negatively affected the seed germination.

Table 4.3: The percentage of seed germination of *Mucuna bracteata* under different hydropriming conditions

Imbibition period (h)	Volume of sdH_2O to moisten cotton layer (mL)	Percentage of seed germination (%)			Seed vigor index
		Day 4	Day 8	Day 12	
0	0	$0.0 \pm 0.0_{a}$	$0.0 \pm 0.0_{a}$	$0.0 \pm 0.0_{a}$	0
	10	$0.0 \pm 0.0_{a}$	$33.0 \pm 3.8_{ef}$	$42.0 \pm 4.0_{e}$	2463
	15	$0.0 \pm 0.0_{a}$	$24.0 \pm 6.2_{cdef}$	$31.0 \pm 4.4_{de}$	1814
	40	$1.1 \pm 1.1_{a}$	$19.0 \pm 7.0_{bcd}$	$23.0 \pm 6.2_{cd}$	1035
6	0	$0.0 \pm 0.0_{a}$	$0.0 \pm 0.0_{a}$	$0.0 \pm 0.0_{ab}$	0
	10	$8.9 \pm 4.4_{abc}$	$37.0 \pm 3.8_{f}$	$43.0 \pm 3.3_{e}$	2473
	15	$11.0 \pm 1.1_{bcd}$	$33.0 \pm 5.1_{ef}$	$39.0 \pm 8.0_{e}$	2067
	40	$2.2 \pm 1.1_{ab}$	$14.0 \pm 1.2_{bd}$	$17.0 \pm 1.9_{bc}$	1339
24	0	$2.9 \pm 1.6_{ab}$	$8.3 \pm 1.9_{abc}$	$9.3 \pm 2.1_{ab}$	465
	10	$12.0 \pm 6.2_{bcd}$	$27.0 \pm 2.2_{def}$	$33.0 \pm 2.8_{de}$	2310
	15	$12.0 \pm 7.4_{bcd}$	$37.0 \pm 6.0_{f}$	$39.0 \pm 5.0_{e}$	1950
	40	$9.1 \pm 3.1_{abc}$	$12.0 \pm 4.8_{abc}$	$14.0 \pm 6.6_{bc}$	700
48	0	$9.4 \pm 2.8_{abc}$	$22.0 \pm 3.5_{bcde}$	$24.0 \pm 2.9_{cd}$	120
	10	$17.0 \pm 5.5_{cd}$	$27.0 \pm 3.9_{def}$	$32.0 \pm 4.0_{de}$	2320
	15	$21.0 \pm 1.1_{d}$	$33.0 \pm 5.9_{ef}$	$34.0 \pm 5.3_{de}$	2720
	40	$18.0 \pm 3.4_{cd}$	$34.0 \pm 5.2_{bcde}$	$21.0 \pm 4.7_{bcd}$	1750

*Mean ± standard error values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p<0.05$

Despite having the same germination rate, the seed vigor index for seeds imbibed for 6 h and cultured on wet cotton roll with 10 mL of sdH_2O (2473) was higher than that of seeds without imbibition and cultured on wet cotton roll with similar volume of sdH_2O (2463) (Table 4.3).

M. bracteata seeds were also imbibed in an osmopriming agent to investigate their effect on seed germination (Section 3.5.3). The study showed that 55% of seeds germinated in 10% (w/v) PEG 6000 solution, whereas only 33% seeds germinated in sdH_2O (Table 4.4).

The study also investigated the effects of combining hydropriming with 2.27 mM ascorbic acid or 0.1% (w/v) activated charcoal to promote the seed germination (Section 3.5.3). The percentage of germinated seeds on 0.1% (w/v) activated charcoal and 2.27 mM ascorbic acid were found to be 44.0% and 39.0%, respectively, albeit significantly (p<0.05) lower than osmopriming treatment (55.0%) (Table 4.4). However, the seed vigor index was highest in seeds germinated on 0.1% (w/v) activated charcoal (3740) compared to osmopriming treatment (2946) or hydropriming treatment alone (1898).

Table 4.4: The percentage of seed germination of *Mucuna bracteata* under osmopriming and different antioxidant treatments

Treatment	Percentage of seed germination (%)			Seed vigor index
	Day 4	Day 8	Day 12	
sdH_2O	$9.0 \pm 4.5_a$	$27.0 \pm 4.9_a$	$33.0 \pm 5.0_a$	1898
PEG	$17.0 \pm 2.6_{ab}$	$42.0 \pm 2.9_b$	$55.0 \pm 3.9_b$	2946
2.27 mM ascorbic acid	$20.0 \pm 1.7_b$	$30.0 \pm 4.9_{ab}$	$39.0 \pm 4.4_a$	2640
0.1% (w/v) activated charcoal	$20.0 \pm 1.7_b$	$40.0 \pm 3.0_{ab}$	$44.0 \pm 2.7_{ab}$	3740

*Mean ± standard error values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at p<0.05

4.4.4 Seeds germination conditions

The effect of photoperiods and culture methods on the seed germination and development were also investigated (Section 3.5.4) (Section 3.3.2). Scarified seeds were cultured on either MS media or wet cotton roll and incubated in the dark or under 16 h light regime. The study showed that the percentage of germinated seeds in the dark condition (36.4-36.9% at Day 12) was higher compared to under light exposure (23.6-31.7% at Day 12) (Table 4.5). Culturing the seeds on either on either MS media or wet cotton roll did not significantly affect the seed germination.

Table 4.5: The percentage of seed germination of *Mucuna bracteata* in different culture conditions

Light regime	Culture method	Percentage of seed germination (%)		
		Day 4	Day 8	Day 12
Dark	MS media	$11.4 \pm 4.31_a$	$30.8 \pm 5.10_a$	$36.4 \pm 6.30_a$
	Cotton roll	$17.5 \pm 6.61_a$	$34.4 \pm 9.60_a$	$36.9 \pm 8.50_a$
Light	MS media	$3.3 \pm 2.20_a$	$11.7 \pm 8.20_a$	$23.6 \pm 5.20_a$
	Cotton roll	$6.7 \pm 4.41_a$	$24.2 \pm 5.80_a$	$31.7 \pm 7.90_a$

*Mean ± standard error values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p<0.05$

4.4.5 Shoot induction

In this study, leaf discs, nodal segments, and cotyledonary nodes were cultured on MS media supplemented with different concentrations of BAP (4.44–17.76 μM) and kinetin (4.65–18.59 μM) (Section 3.5.5).

The results demonstrated that cotyledonary nodes on MS media containing 4.44 μM BAP alone and in combination with 4.65 μM kinetin were significantly different compared to other treatments, as they induced the highest number of shoots per explants (4.83-5.60) (Table 4.6). In comparison, MS media without supplementation of plant growth regulators produced longer shoots than other treatments. No shoot was formed from the leaf discs in any treatment after two months of culture (Figure 4.16). The highest percentage of callus formation in leaf discs was 48.3% on MS media supplemented with 17.8 μM BAP (Table 4.6). Cotyledonary nodes were found to be more effective than nodal segments and leaf discs for shoot initiation when cultured on MS media supplemented with cytokinin at low concentration (Table 4.6) (Figure 4.17).

Table 4.6: Effect of different concentrations of BAP and kinetin on shoot multiplication of *Mucuna bracteata*

Cytokinin (µM)		Cotyledonary nodes		Nodal segments		Leaf
BAP	Kinetin	Number shoots per explants	Shoot length (cm)	Number shoots per explants	Shoot length (cm)	Callus formation (%)
-	-	$1.43 \pm 0.03_{a}$	$14.36 \pm 2.05_{d}$	$1.04 \pm 0.04_{a}$	$2.79 \pm 0.60_{ab}$	$0.0 \pm 0.00_{a}$
4.44	-	$5.60 \pm 0.20_{e}$	$6.45 \pm 0.42_{c}$	$1.42 \pm 0.14_{bcd}$	$3.84 \pm 0.55_{b}$	$23.9 \pm 5.10_{cd}$
8.88	-	$3.63 \pm 0.41_{d}$	$3.89 \pm 0.27_{ab}$	$1.41 \pm 0.13_{bcd}$	$2.70 \pm 0.46_{ab}$	$35.0 \pm 5.27_{d}$
13.3	-	$1.90 \pm 0.20_{ab}$	$2.86 \pm 0.34_{a}$	$1.50 \pm 0.19_{d}$	$3.31 \pm 0.33_{ab}$	$46.9 \pm 5.58_{e}$
17.8	-	$2.91 \pm 0.07_{cd}$	$2.95 \pm 0.22_{a}$	$1.16 \pm 0.07_{abc}$	$2.46 \pm 0.31_{ab}$	$48.3 \pm 5.33_{e}$
4.44	4.65	$4.83 \pm 0.64_{e}$	$6.27 \pm 0.38_{c}$	$1.46 \pm 0.14_{cd}$	$3.28 \pm 0.31_{ab}$	$21.3 \pm 4.76_{c}$
-	4.65	$1.93 \pm 0.12_{ab}$	$5.37 \pm 0.54_{bc}$	$0.94 \pm 0.06_{a}$	$1.84 \pm 0.53_{a}$	$7.7 \pm 7.69_{a}$
-	9.29	$1.80 \pm 0.20_{ab}$	$7.24 \pm 0.79_{c}$	$0.94 \pm 0.06_{a}$	$3.03 \pm 0.60_{ab}$	$23.9 \pm 6.36_{bc}$
-	13.9	$1.52 \pm 0.07_{a}$	$7.40 \pm 1.32_{c}$	$1.11 \pm 0.06_{ab}$	$3.18 \pm 0.54_{ab}$	$9.8 \pm 4.21_{ab}$
-	18.6	$2.57 \pm 0.46_{bc}$	$5.48 \pm 0.57_{bc}$	$1.04 \pm 0.04_{a}$	$3.77 \pm 0.53_{b}$	$20.0 \pm 5.21_{bc}$

*Mean ± standard error values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p<0.05$

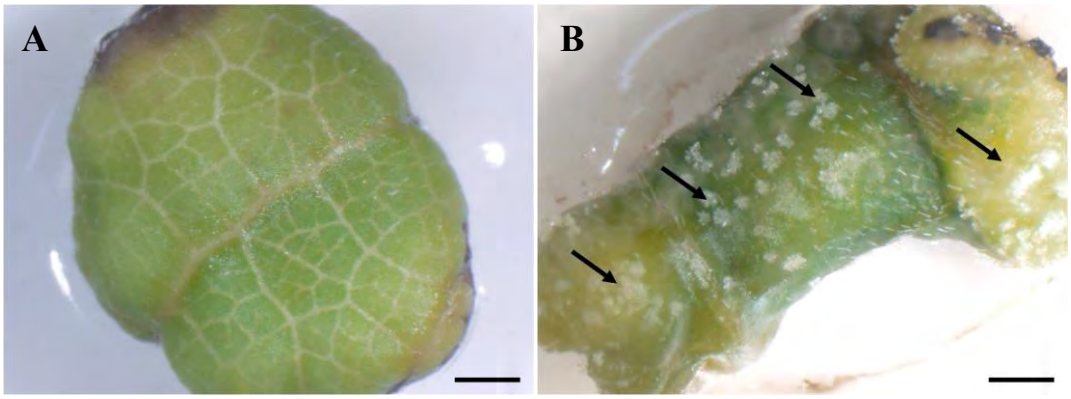


Figure 4.16: Callus formation from leaf discs of *Mucuna bracteata* cultured on MS media supplemented with different concentrations of BAP and kinetin. A: Representative of explants without any callus formation; B: Representative of explants with callus formation (black arrows indicated region of callus formation. Bar = 1 mm.

Figure 4.17: Shoot multiplication from different explants of *Mucuna bracteata* cultured on MS media supplemented with 4.44 µM BAP. A: Cotyledonary node; B: Nodal segment. Bar = 1 cm.

4.4.6 Root induction

Elongated shoots (5-20 cm in length) from Section 4.4.5 were cultured on MS media supplemented with different concentrations of NAA (2.69–16.1 μM) to induce root formation (Section 3.5.6). Shoots of at least 5 cm in height started to root after two weeks of culture (Figure 4.18). Cotyledonary node-derived shoots produced the highest percentage rooting response (92.9%) with a mean of 1.38 roots per shoot and a mean length of 4.55 cm after one month of culture on MS media supplemented with 10.7 μM NAA (Table 4.7). Only 43.3% nodal segment-derived shoots developed roots after one month of culture on MS media containing 16.1 μM NAA.

Figure 4.18: Root induction from plantlet cultured on MS media supplemented with NAA. Bar = 1 cm.

Table 4.7: Effect of different concentrations of NAA on root induction of *Mucuna bracteata*

NAA (μM)	Cotyledonary nodes			Nodal segments		
	Percentage of shoots forming roots (%)	Number of roots per shoot	Root length (cm)	Percentage of shoots forming roots (%)	Number of roots per shoot	Root length (cm)
0.0	$36.7 \pm 3.33_{a}$	$1.30 \pm 0.23_{a}$	$7.06 \pm 1.46_{a}$	$3.33 \pm 3.33_{a}$	$1.00 \pm 0.00_{a}$	$2.00 \pm 0.00_{a}$
2.69	$78.9 \pm 6.76_{b}$	$1.73 \pm 0.09_{a}$	$5.59 \pm 0.55_{ab}$	$10.0 \pm 0.00_{a}$	$1.67 \pm 0.67_{a}$	$8.94 \pm 2.40_{b}$
5.37	$90.5 \pm 2.38_{b}$	$1.71 \pm 0.06_{a}$	$4.61 \pm 0.16_{ab}$	$6.67 \pm 3.33_{a}$	$1.00 \pm 0.00_{a}$	$3.75 \pm 2.25_{ab}$
10.7	$92.9 \pm 0.00_{b}$	$1.38 \pm 0.09_{a}$	$4.55 \pm 0.12_{ab}$	$20.0 \pm 0.00_{b}$	$1.33 \pm 0.17_{a}$	$4.33 \pm 0.82_{ab}$
16.1	$89.5 \pm 6.75_{b}$	$1.45 \pm 0.10_{a}$	$4.21 \pm 0.67_{b}$	$43.3 \pm 3.33_{c}$	$3.20 \pm 0.30_{b}$	$6.50 \pm 0.59_{ab}$

*Mean ± standard error values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p<0.05$

4.4.7 Acclimatization

A total of 100 rooted plantlets were transplanted in polybags containing garden soil and maintained in the greenhouse. Survival of 89% of the plantlets with uniform growth was recorded after four weeks of transplanting (Figure 4.19).

Figure 4.19: Acclimatized plantlets of *Mucuna bracteata* in greenhouse condition after four weeks. Bar = 10 cm.

4.4.8 Simple sequence repeats (SSR) analysis

SSR analysis was performed to evaluate the genetic fidelity of the *in vitro*-derived plants compared to seed-germinated plants (Section 3.5.7). Out of ten SSR primers tested (Appendix B: Table 2), only three primers did not produced bands. These primers were CBT 02, RM 154 and RM205. The bands appeared to be monomorphic across all the plants, indicating the clonal fidelity of *in vitro*-derived plants (Figure 4.20).

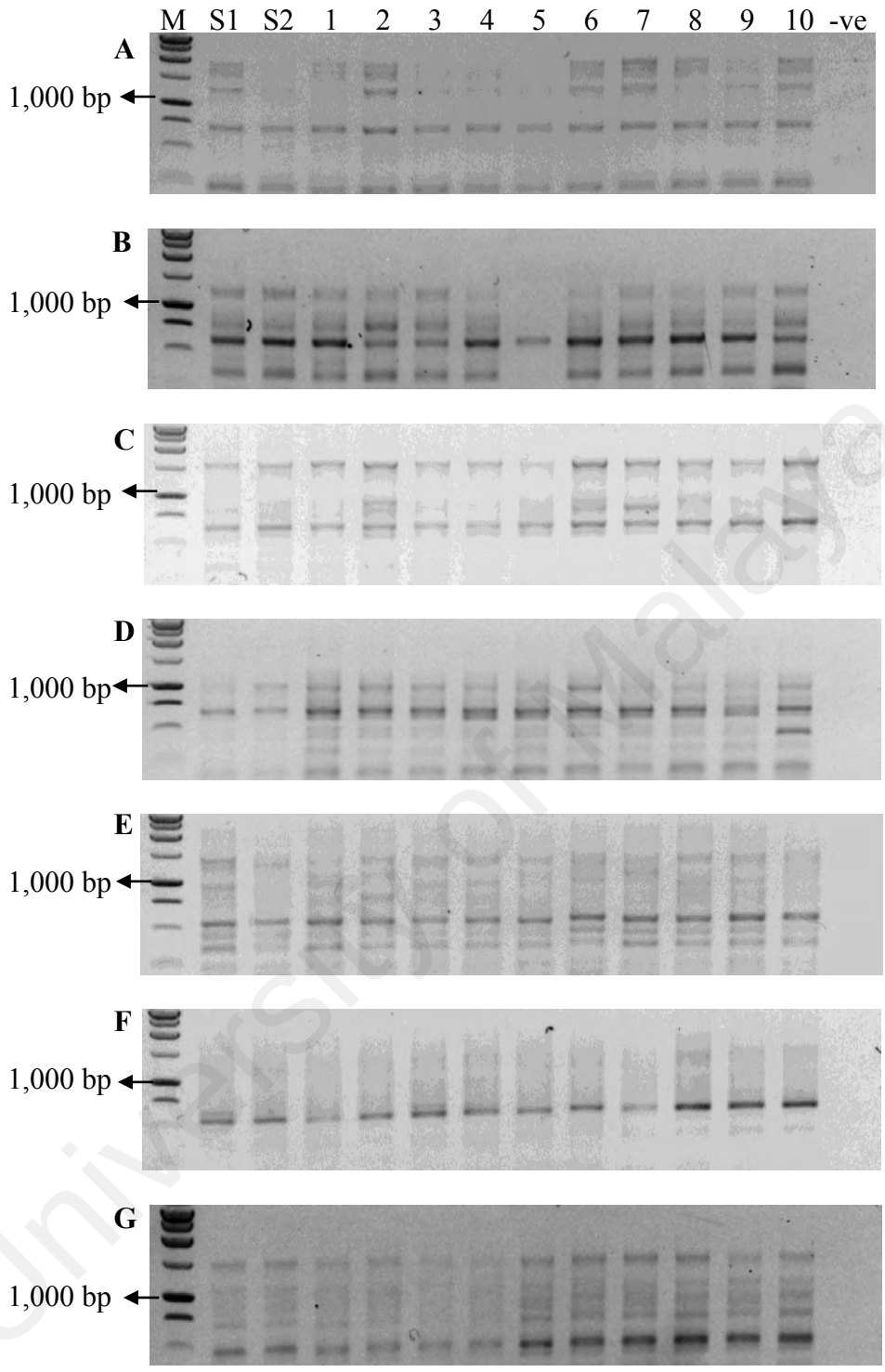


Figure 4.20: Representative image for SSR analysis using (A) CBT 03 primer, (B) CBT 04 primer, (C) CBT 07 primer, (D) RM 1 primer, (E) RM 72 primer, (F) RM 135 primer and (G) RM 287 primer. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane S1 and Lane S2: seed-germinated plants; Lanes 1-10: *in vitro*-derived plants; Lane –ve: PCR negative control using sdH_2O as DNA template.

4.4.9 DNA content and genome size estimation

DNA content and genome size estimation for *M. bracteata* based on the fluorescence intensity of soybean as standard was conducted using flow cytometry analysis (Section 3.5.8). The analysis also revealed that *in vitro*-derived and seed-germinated plants had about similar fluorescence intensity, suggesting the ploidy stability among the *in vitro*-derived plants. The mean peak of fluorescence intensity for standard, *in vitro*-derived and seed-germinated plants was 204.5 (Figure 4.21A), 121.2 ± 0.83 (Figure 4.21B) and 123.1 ± 2.19 (Figure 4.21C), respectively, with coefficients of variation (CV) within an acceptable range of 1.33 % to 4.70 %. The DNA content of *M. bracteata* was estimated to be 1.48 ± 0.01 pg based on the fluorescence intensity of the *in vitro*-derived plants and the projected genome size of *M. bracteata* is about 1,448 ± 9 Mb.

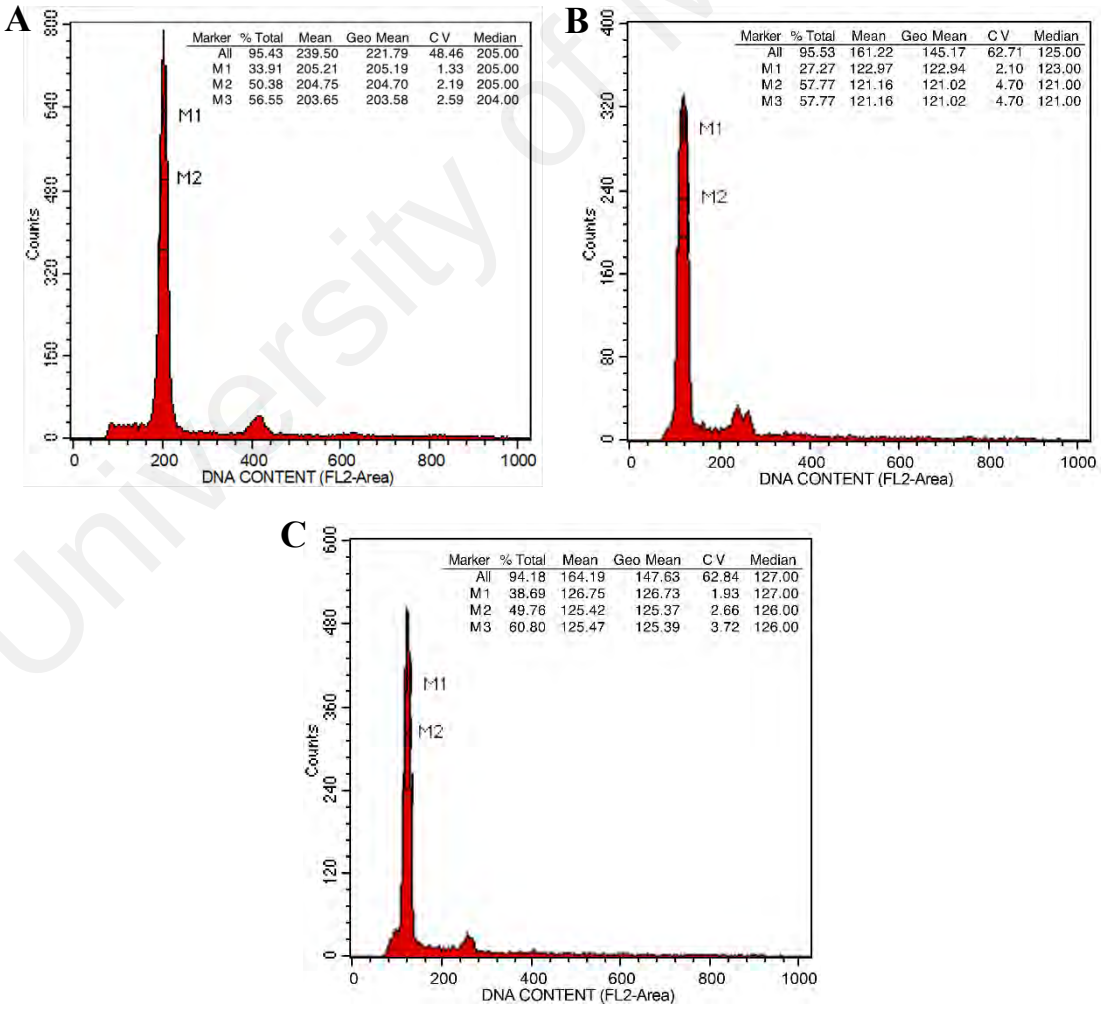


Figure 4.21 Representative flow cytometry histogram of relative fluorescence intensity of propidium iodine-stained nuclei. A: Soybean; B: *In vitro*-derived *Mucuna bracteata* plant; B: seed-derived *Mucuna bracteata* plant.

4.5 Transient expression of anti-toxoplasma IgG in *Mucuna bracteata*

4.5.1 Comparison between syringe infiltration and vacuum-assisted infiltration

In order to determine the best method of infiltration for *M. bracteata*, leaves from five week-old plant were either syringe infiltrated (Section 3.6.2) or vacuum-assisted infiltrated (Section 3.6.3) with *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 (Section 3.6.1). Successful infiltration was indicated by the presence of dark green patches in the leaves. These dark green patches were absent in non-infiltrated leaves (Figure 4.22A).

In syringe infiltration, extreme caution was practiced as the leaves of *M. bracteata* were brittle and easily damaged when too much pressure was applied. Nonetheless, the infiltrated area did not expand beyond the syringe nozzle area (Figure 4.22B). In vacuum-assisted infiltration, many erratic dark green patches were observed throughout the infiltrated leaves (Figure 4.22C).

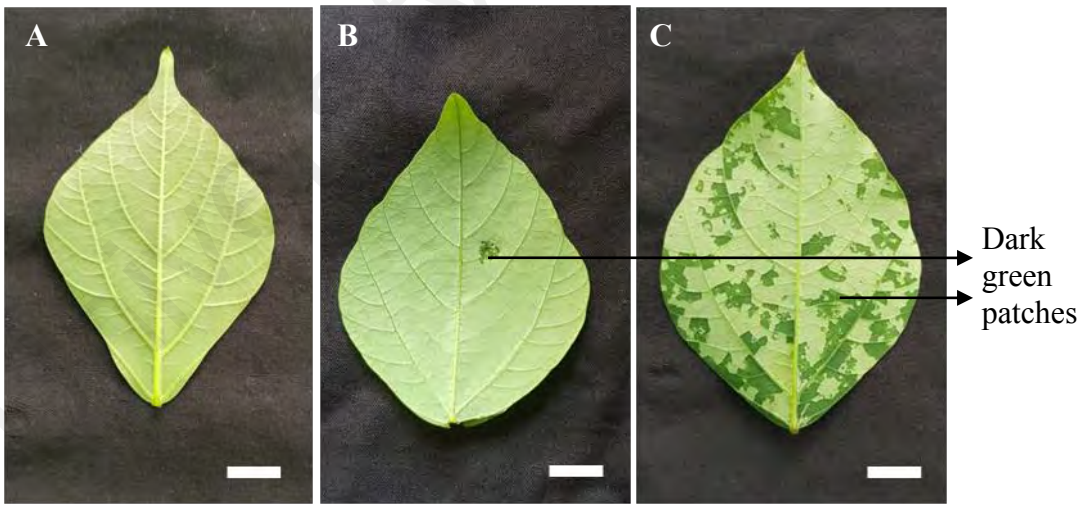


Figure 4.22: Comparison between syringe infiltration and vacuum-assisted infiltration of *Mucuna bracteata*. A: Non-infiltrated leaflet of *M. bracteata*. B: Syringe-infiltrated leaflet of *M. bracteata*. C: Vacuum-assisted infiltrated leaflet of *M. bracteata*. Bar = 1 cm.

4.5.2 Optimization of selected parameters in vacuum-assisted infiltration

4.5.2.1 Trifoliate position

Five week-old *M. bracteata* plants were infiltrated with 1 L of infiltration buffer containing *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 (Section 3.6.1) as described in Section 3.6.3. Anti-toxoplasma IgG from bottom, intermediate and top trifoliate leaves that were harvested at 2 days post infiltration were extracted using PBS and quantitated (Section 3.4.1 – Section 3.4.5).

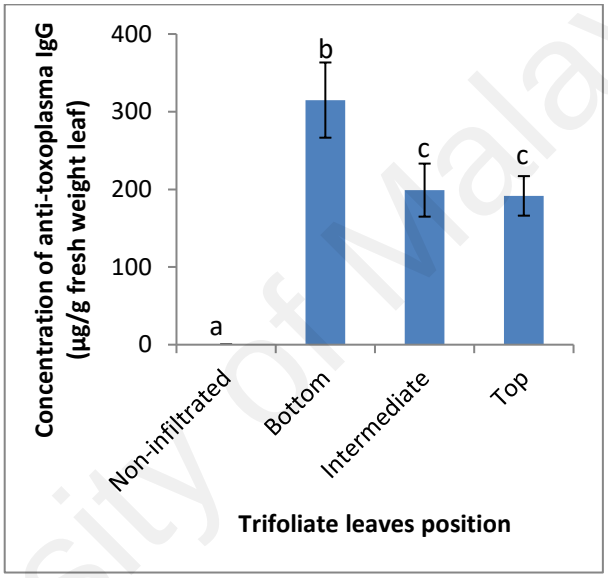


Figure 4.23: Effect of *Mucuna bracteata* trifoliate leaves position on anti-toxoplasma IgG concentration. The bars indicate mean ± standard errors. Mean followed by the same letters are not statistically significant according to Duncan's multiple-range analysis at $p < 0.05$.

In this study, the bottom trifoliate leaves produced ~1.5 fold higher concentration of anti-toxoplasma IgG (314.9 ± 49.8 µg/g fresh weight leaf) compared to trifoliate leaves at other positions (Figure 4.23). There was no significant difference between the intermediate (199.0 ± 31.2 µg/g fresh weight leaf) and the top position (191.6±24.5 µg/g fresh weight leaf).

4.5.2.2 Post infiltration period before harvesting

Five week-old *M. bracteata* plants were infiltrated with 1 L of infiltration buffer containing *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 (Section 3.6.1) as described in Section 3.6.3. Anti-toxoplasma IgG from bottom trifoliate leaves that were harvested at 2, 4 and 6 days post infiltration were extracted using PBS and quantitated (Section 3.4.1 – Section 3.4.5).

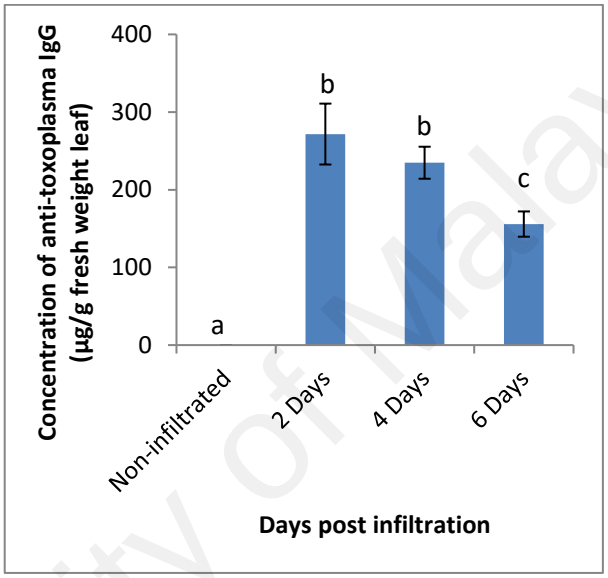


Figure 4.24: Concentration of anti-toxoplasma IgG at different days post infiltration. The bars indicate mean ± standard errors. Mean followed by the same letter are not statistically significant according to Duncan's multiple-range analysis at $p< 0.05$.

The results showed that concentrations of anti-toxoplasma IgG at two days (271.68 ± 39.2 µg/g fresh weight leaf) and four days (234.78 ± 20.6 µg/g fresh weight leaf) post infiltration were significantly higher compared to six days post infiltration (155.8 ± 16.3 µg/g fresh weight leaf) (Figure 4.24).

4.5.2.3 *Agrobacterium* strains

Five week-old *M. bracteata* plants were infiltrated with 1 L of infiltration buffer containing *A. tumefaciens* strain GV3101, EHA105 or LBA4404 harboring pTRAkcHcLcTg130 (Section 3.6.1) as described in Section 3.6.3. Anti-toxoplasma IgG from bottom trifoliate leaves that were harvested at 2 days post infiltration were extracted using PBS and quantitated (Section 3.4.1 – Section 3.4.5).

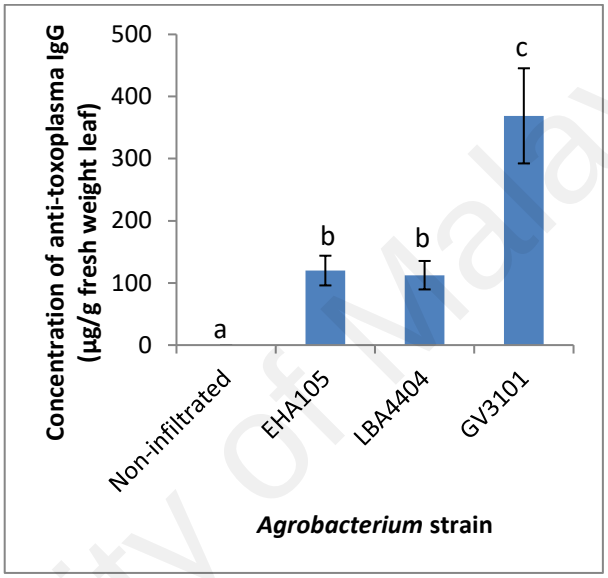


Figure 4.25: Effect of different *Agrobacterium* strains on anti-toxoplasma IgG concentration. The bars indicate mean ± standard errors. Mean followed by the same letter are not statistically significant according to Duncan's multiple-range analysis at $p < 0.05$.

The results demonstrated that the highest concentration of anti-toxoplasma IgG was observed when *M. bracteata* was infiltrated with *A. tumefaciens* strain GV3101 (368.92 ± 76.6 µg/g fresh weight leaf) (Figure 4.25). In contrast, the concentration of anti-toxoplasma IgG decreased threefold when *M. bracteata* was infiltrated with either *A. tumefaciens* strain EHA105 (119.90 ± 23.9 µg/g fresh weight leaf) or LBA4404 (112.46 ± 22.9 µg/g fresh weight leaf).

4.5.2.4 Protein extraction buffer

Five week-old *M. bracteata* plants were infiltrated with 1 L of infiltration buffer containing *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 (Section 3.6.1) as described in Section 3.6.3. Anti-toxoplasma IgG from the bottom trifoliate leaves that were harvested at 2 days post infiltration were extracted using either PBS or NEB and quantitated (Section 3.4.1 – Section 3.4.5).

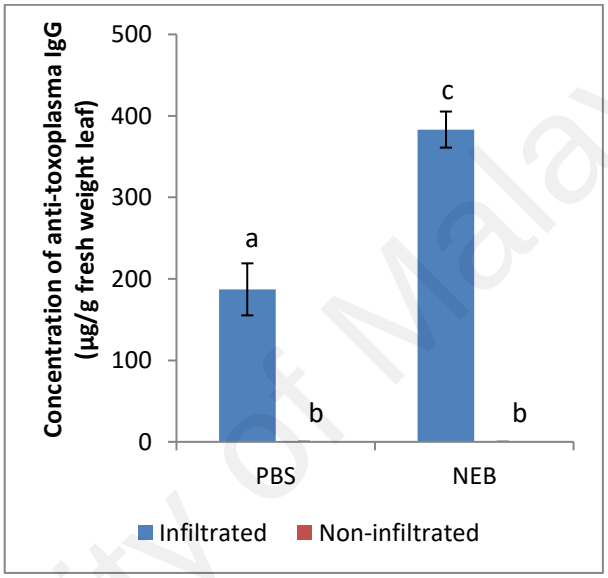


Figure 4.26: Effect of different buffers for crude protein extraction on anti-toxoplasma IgG concentration. The bars indicate mean ± standard errors. Mean followed by the same letter are not statistically significant according to Duncan's multiple-range analysis at $p < 0.05$.

In this study, the concentration of anti-toxoplasma IgG for extraction using NEB (383.19 ± 22.1 µg/g fresh weight leaf) was twofold higher than extraction using PBS (181.14 ± 31.9 µg/g fresh weight leaf) (Figure 4.26). In addition, it was observed that crude protein extraction using PBS often yield reddish brown crude extract that sometimes precipitated after half an hour even when frozen or maintained at 4 ^{o}C. On the other hand, crude protein extraction using NEB produced green protein extract that did not precipitate.

4.5.3 Anti-toxoplasma IgG concentration under optimal infiltration condition

Based on the optimal infiltration conditions (Section 4.5.2.1 – Section 4.5.4), five week-old *M. bracteata* plants were infiltrated with 1 L of infiltration buffer (Appendix C9) containing *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 (Section 3.6.1) as described in Section 3.6.3. The bottom trifoliate leaves from vacuum-assisted infiltrated and non-infiltrated *M. bracteata* plants were harvested at 2 days post infiltration. Crude protein was extracted using NEB and anti-toxoplasma IgG was purified and quantitated as described in Section 3.4.1 – Section 3.4.5.

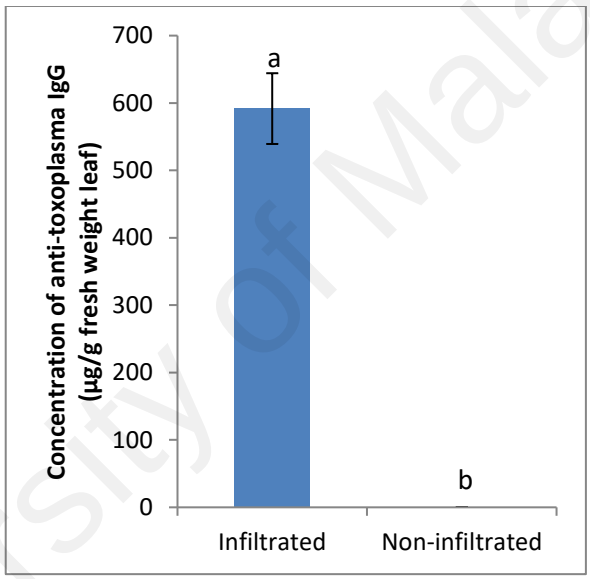


Figure 4.27: Anti-toxoplasma IgG concentration between vacuum-assisted infiltrated and non-infiltrated leaves of *Mucuna bracteata*. The bars indicate mean ± standard errors. Mean followed by the same letter are not statistically significant according to Duncan's multiple-range analysis at $p < 0.05$.

Under the optimal infiltration conditions, *M. bracteata* produced 591.10 ± 52.5 µg/g fresh weight leaf of anti-toxoplasma IgG (Figure 4.27). As expected, no anti-toxoplasma IgG was detected from non-infiltrated *M. bracteata*.

4.5.4 Western blot analysis

Anti-toxoplasma IgG that was extracted and purified (Section 3.4.1 – Section 3.4.6) from *M. bracteata* (Section 3.6.3) was analyzed via Western blot using anti-human IgG Fab fragment antibody (Section 3.4.7). A band with an estimated molecular mass of ~58 kDa was detected in the infiltrated leaves (Figure 4.28). However, an additional band with an estimated molecular mass of ~51 kDa was also observed in the infiltrated leaves (Figure 4.28). As expected, no band was detected in the non-infiltrated leaves.

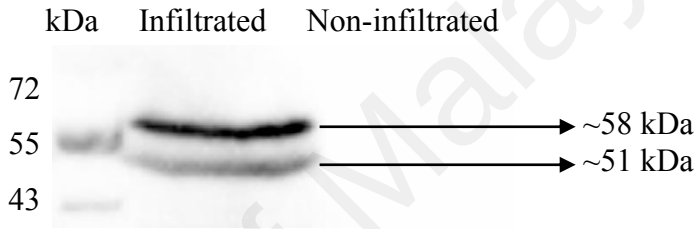


Figure 4.28: Western blot analysis of purified anti-toxoplasma IgG from vacuum-assisted infiltrated and non-infiltrated leaves of *Mucuna bracteata*. EZRun-Prestained Rec Protein Ladder (Fisher Bioreagents, USA) was used as marker.

4.5.5 ELISA

The antigen binding property of anti-toxoplasma IgG extracted and purified (Section 3.4.1 – Section 3.4.6) from *M. bracteata* (Section 3.6.3) was analyzed via ELISA (Section 3.4.8). Detection of biological activity in the infiltrated leaves (3.14 ± 0.44 U/mL) (Figure 4.29) indicated that the antibody retained its antigen binding properties. As expected, no biological activity was detected in the non-infiltrated leaves.

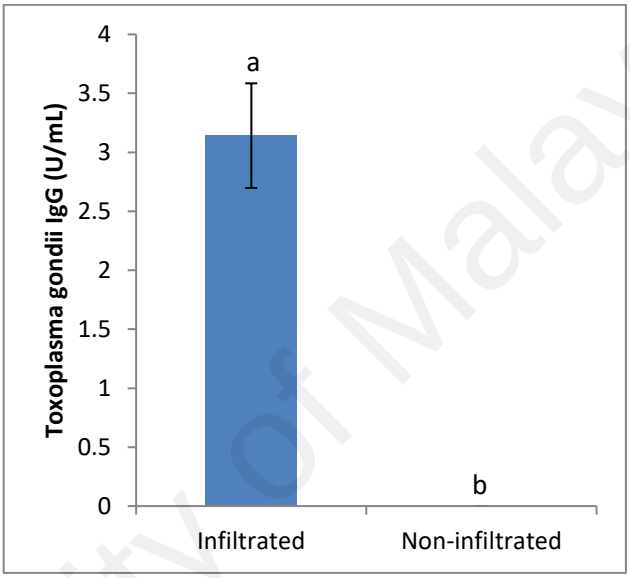


Figure 4.29: ELISA analysis of purified anti-toxoplasma IgG from vacuum-assisted infiltrated and non-infiltrated leaves of *Mucuna bracteata*. Analysis was carried out using Human Anti-*Toxoplasma gondii* IgG ELISA kit (Abcam, UK) according to the manufacturer's protocol. The bars indicate mean ± standard errors. Mean followed by the same letter are not statistically significant according to Duncan's multiple-range analysis at $p < 0.05$.

4.6 Transient expression of anti-toxoplasma IgG in *Nicotiana benthamiana*

4.6.1 Anti-toxoplasma IgG concentration

Using the established method of Leuzinger et al. (2013), the sixth leaf of 40 day-old *N. benthamiana* plant was syringe infiltrated with infiltration buffer containing *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 (Section 3.6.1) (Section 3.6.4). The infiltrated leaves were harvested at 6 days post infiltration. Non-infiltrated leaves were also harvested as negative controls. Crude protein was extracted using NEB and anti-toxoplasma IgG was purified and quantitated as described in Section 3.4.1 – Section 3.4.5.

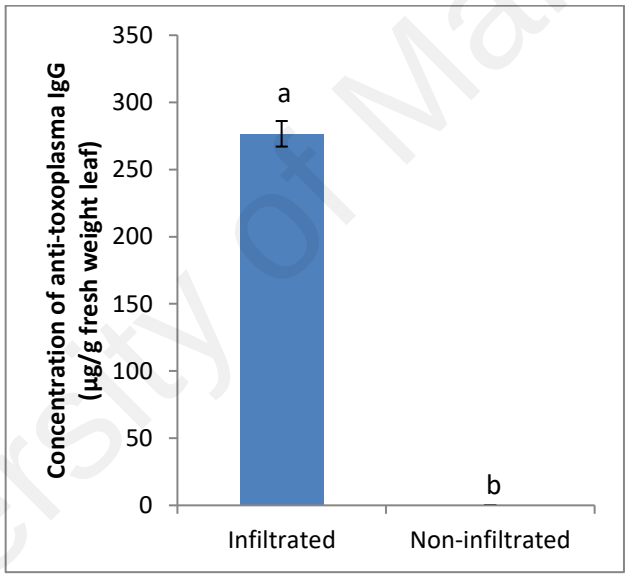


Figure 4.30: Anti-toxoplasma IgG concentration between syringe infiltrated and non-infiltrated leaves of *Nicotiana benthamiana*. The bars indicate mean ± standard errors. Mean followed by the same letter are not statistically significant according to Duncan's multiple-range analysis at $p < 0.05$.

The concentration of anti-toxoplasma IgG in syringe infiltrated leaves of *N. benthamiana* was determined to be 276.61 ± 9.52 µg/g fresh weight leaf (Figure 4.30). As expected, no anti-toxoplasma IgG was quantitated from non-infiltrated leaves.

4.6.2 Western blot analysis

Anti-toxoplasma IgG that was extracted and purified (Section 3.4.1 – Section 3.4.6) from *N. benthamiana* (Section 3.6.4) was analyzed via Western blot using anti-human IgG Fab fragment antibody (Section 3.4.7). A band with an estimated molecular mass of ~58 kDa was detected in the infiltrated leaves (Figure 4.31). As expected, no band was detected in the non-infiltrated leaves.

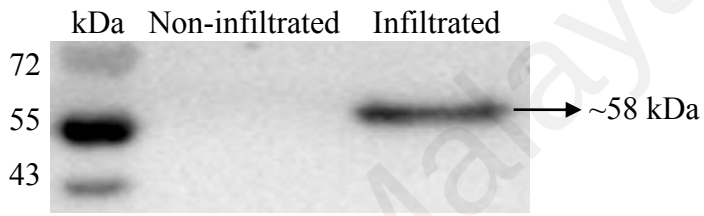


Figure 4.31: Western blot analysis of purified anti-toxoplasma IgG from syringe infiltrated and non-infiltrated leaves of *Nicotiana benthamiana.* EZRun-Prestained Rec Protein Ladder (Fisher Bioreagents, USA) was used as marker.

4.6.3 ELISA

The antigen binding property of anti-toxoplasma IgG extracted and purified (Section 3.4.1 – Section 3.4.6) from *N. benthamiana* (Section 3.6.4) was analyzed via ELISA (Section 3.4.8). Detection of biological activity in the infiltrated leaves (3.30 ± 0.81 U/mL) (Figure 4.32) indicated that the antibody retained its antigen binding properties. As expected, no biological activity was detected in the non-infiltrated leaves.

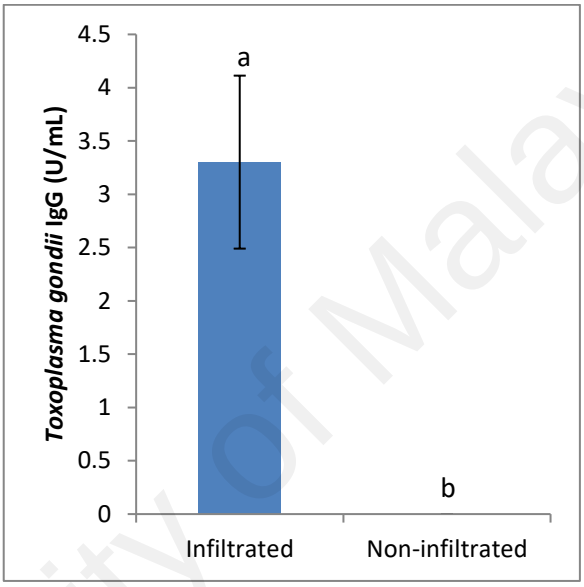


Figure 4.32: ELISA analysis of purified anti-toxoplasma IgG from syringe infiltrated and non-infiltrated leaves of *Nicotiana benthamiana*. Analysis was carried out using Human Anti-*Toxoplasma gondii* IgG ELISA kit (Abcam, UK) according to the manufacturer's protocol. The bars indicate mean ± standard errors. Mean followed by the same letter are not statistically significant according to Duncan's multiple-range analysis at $p < 0.05$.

4.7 Optimization of stable transformation parameters in *Mucuna bracteata*

4.7.1 Natural regeneration capacity of *Mucuna bracteata* cotyledonary node under antibiotic selection pressure

4.7.1.1 Shoot regeneration capacity

Shoot regeneration capacity is the ability of *M. bracteata* cotyledonary node explants to generate shoots when cultured on MS media supplemented with 4.44 µM of BAP and various concentration of kanamycin (50-150 µg/mL) or G418 disulphate (12.5-75 µg/mL) (Section 3.7.2.1). Percentage of explants with shoot formation was recorded after one month of culture and presented in Figure 4.33.

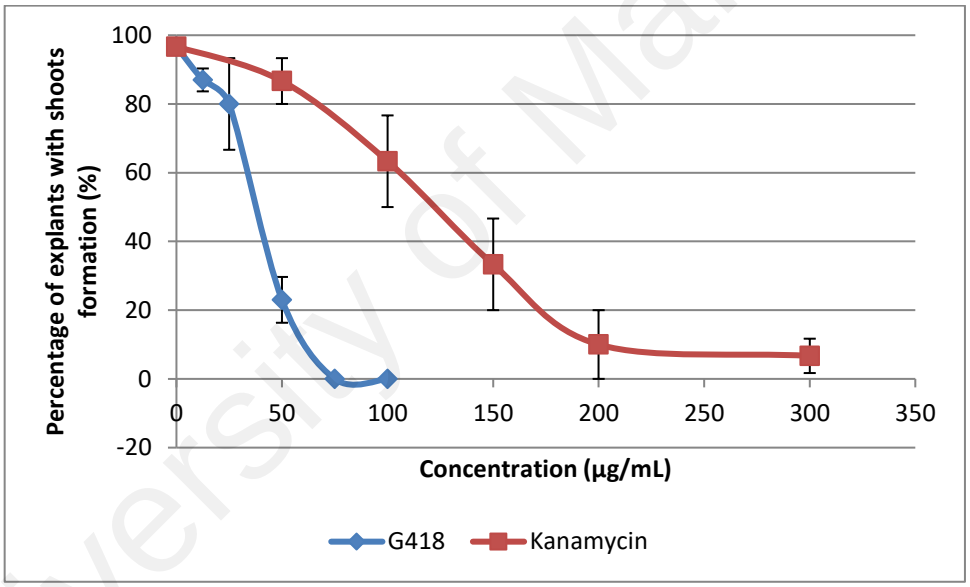


Figure 4.33: Shoot regeneration capacity of *Mucuna bracteata* cotyledonary explants on MS media supplemented with 4.44 µM of BAP and various concentration of kanamycin or G418 disulphate.

Increasing concentration of either kanamycin or G418 disulphate in the media led to decreasing percentage of explants with shoots formation (Figure 4.33). Similarly, the average number of shoot per explants responding and the average height of shoot also decreased with increasing concentration of either kanamycin or G418 disulphate (Table 4.8). The results demonstrated that *M. bracteata* cotyledonary explants were more sensitive to G418 disulphate compared to kanamycin. Shoot formation was completely arrested when the MS media was supplemented with 75 µg/mL of G418 disulphate (Figure 4.33) (Table 4.8). However, shoot formation was not completely arrested even when the MS media was supplemented with kanamycin at concentration of 300 µg/mL (Figure 4.33) (Table 4.8). Furthermore, the percentage of explants with shoot formation, the average number of shoot per explants responding and the average height of shoot started to plateau when the MS media was supplemented with kanamycin at concentration of 200 µg/mL onward (Figure 4.33) (Table 4.8).

Phenotypic observation of *M. bracteata* cotyledonary explants that were cultured on MS media supplemented with G418 disulphate showed that necrotic lesions could be observed along the shoots and leaflet of shoots regenerated on media that was supplemented with 25 µg/mL and 50 µg/mL of G418 disulphate (Figure 4.34C,D). Necrotic lesions were also observed throughout the cotyledonary explants on media that was supplemented with 50 µg/mL, 75 µg/mL and 100 µg/mL of G418 disulphate (Figure 4.34D,E,F). Even though 23% of the explants that were cultured on media that was supplemented with 50 µg/mL of G418 disulphate were able to generate shoots, these shoots demonstrated abnormal phenotypes (Figure 4.34D). Thus, the concentration of G418 disulphate at 50 µg/mL was selected for the selection of putative transgenic shoots during shoot induction in subsequent experiments (Section 4.7.2) (Section 4.7.3).

Table 4.8: The average number and height of shoots formed from *Mucuna bracteata* cotyledonary explants in the shoot regeneration capacity study

Concentration of antibiotic (µg/mL)		Average number of shoots per explant responding	Average height of shoot (cm)
G418	Kanamycin		
-	-	$5.02 \pm 0.53_{d}$	$4.35 \pm 1.09_{e}$
12.5	-	$3.93 \pm 0.89_{c,d}$	$2.74 \pm 0.40_{b,c,d}$
25	-	$2.01 \pm 0.16_{b}$	$2.45 \pm 0.21_{b,c}$
50	-	$1.78 \pm 0.22_{b}$	$1.79 \pm 0.11_{b}$
75	-	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$
100	-	$0.00 \pm 0.00_{a}$	$0.00 \pm 0.00_{a}$
-	50	$5.06 \pm 0.56_{d}$	$3.98 \pm 0.43_{c,d,e}$
-	100	$2.92 \pm 0.39_{b,c}$	$4.13 \pm 0.12_{d,e}$
-	150	$2.01 \pm 0.35_{b}$	$3.87 \pm 0.38_{c,d,e}$
-	200	$2.05 \pm 0.47_{b}$	$3.55 \pm 0.39_{c,d,e}$
-	300	$1.69 \pm 0.35_{b}$	$3.28 \pm 0.21_{b,c,d,e}$

*Mean ± standard error values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p<0.05$

Figure 4.34: Shoot regeneration capacity of *Mucuna bracteata* cotyledonary explants on MS media supplemented with 4.44 µM of BAP and various concentration of G418 disulphate. A: 0 µg/mL; B: 12.5 µg/mL; C: 25 µg/mL; D: 50 µg/mL; E: 75 µg/mL; F: 100 µg/mL. Bar = 1 cm.

4.7.1.2 Root regeneration capacity

Root regeneration capacity is the ability of *M. bracteata* shoots to generate roots when cultured on MS media supplemented with 10.7 µM of NAA and various concentration of G418 disulphate (12.5-75 µg/mL) (Section 3.7.2.2). Percentage of explants with root formation was recorded after one month of culture and presented in Figure 4.35.

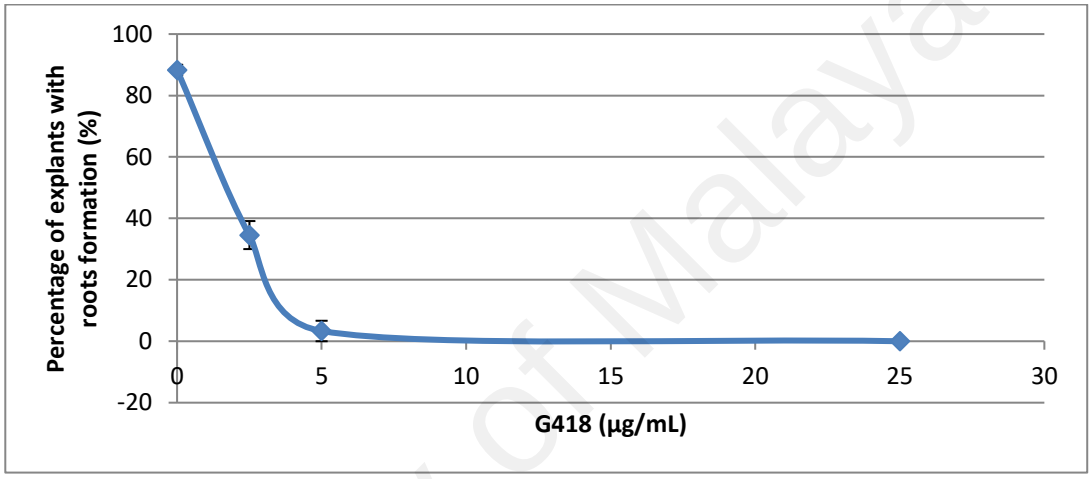


Figure 4.35: Root regeneration capacity of *Mucuna bracteata* shoots on MS media supplemented with 10.7 µM of NAA and various concentration of G418 disulphate.

Increasing concentration of G418 disulphate in the media led to decreasing percentage of explants with root formation (Figure 4.35). Similarly, the average number of root per explants responding and the average length of root also decreased with increasing concentration of G418 disulphate (Table 4.9). The results demonstrated that *M. bracteata* roots formation was highly sensitive towards G418 disulphate. The percentage of explants with roots formation decreased 60% and 96% when the media was supplemented with 2.5 µg/mL and 5 µg/mL of G418 disulphate, respectively.

Table 4.9: The average number and length of roots formed from *Mucuna bracteata* shoot explants in root regeneration capacity study

Concentration of G418 (µg/mL)	Average number of roots per explant responding	Average length of root (cm)
0	$4.13 \pm 1.02_{a}$	$2.97 \pm 0.93_{a}$
2.5	$2.06 \pm 0.06_{a,b}$	$2.01 \pm 0.01_{a,b}$
5	$0.50 \pm 0.50_{b}$	$0.63 \pm 0.53_{b}$
25	$0.00 \pm 0.00_{b}$	$0.00 \pm 0.00_{b}$

*Mean ± standard error values followed by the same letter within a column were not significantly different according to Duncan's multiple-range analysis at $p<0.05$

Even though the average number of root per explants responding and the average length of root decreased significantly when the media was supplemented with G418 disulphate, no necrotic lesion was observed on any of the roots formed (Figure 4.36). However, increasing concentration of G418 disulphate was observed to affect the phenotypes of shoots growth with apparent lesion could be observed on shoots cultured in media supplemented with 25 µg/mL of G418 disulphate. Considering the high sensitivity of *M. bracteata* towards G418 disulphate during rooting formation, concentration of G418 disulphate at 2.5 µg/mL was selected for rooting induction of putative transgenic shoots in subsequent experiments.

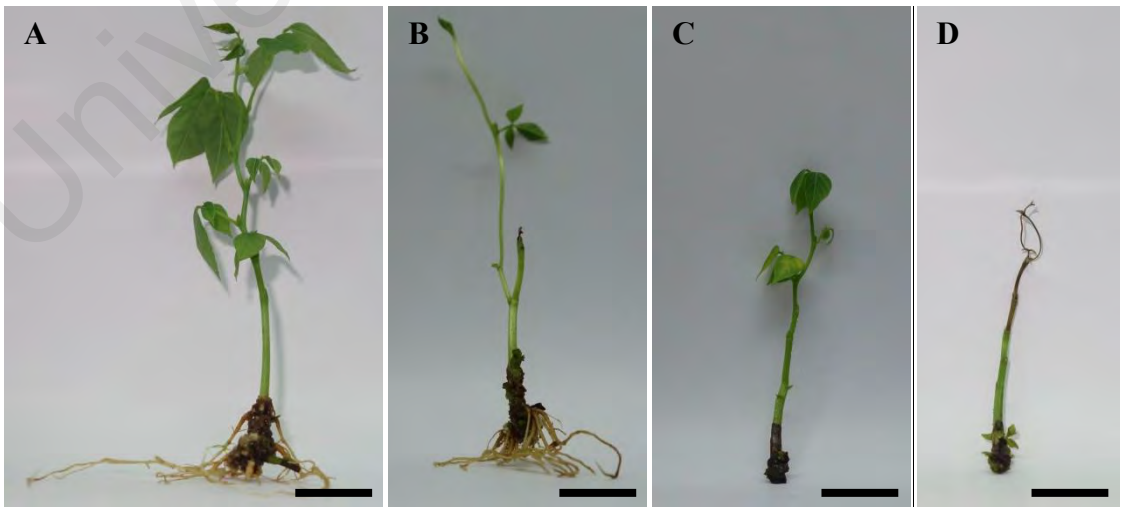


Figure 4.36: Root regeneration capacity of *Mucuna bracteata* shoots on MS media supplemented with 10.7 µM of NAA and various concentration of G418 disulphate. A: 0 µg/mL; B: 2.5 µg/mL; C: 5 µg/mL; D: 25 µg/mL. Bar = 1 cm.

4.7.2 Infection period

Cotyledonary node explants were infected by immersing the explants in *Agrobacterium* suspension for 10, 20 or 30 min (Section 3.7.3.2) and co-cultivated for 2 days (Section 3.7.3.3). The number of shoots generated after one month of culture on MB selection media (Section 3.7.3.4) was recorded (Section 4.7.2.1).

4.7.2.1 Regeneration percentage on MB selection media

After one month of culture on MB selection media, the highest regeneration percentage was recorded for explants that were infected for 20 min (18.43%) whereas the lowest regeneration percentage was recorded for explants that were infected for 30 min (11.11%) (Figure 4.37). However, there was no significance difference observed in the regeneration percentage among all of the infection periods tested. Tissue samples were taken from either the leaves or the shoot tips of regenerants for crude genomic DNA extraction (Section 3.2.2) and PCR analysis (Section 3.7.3.5).

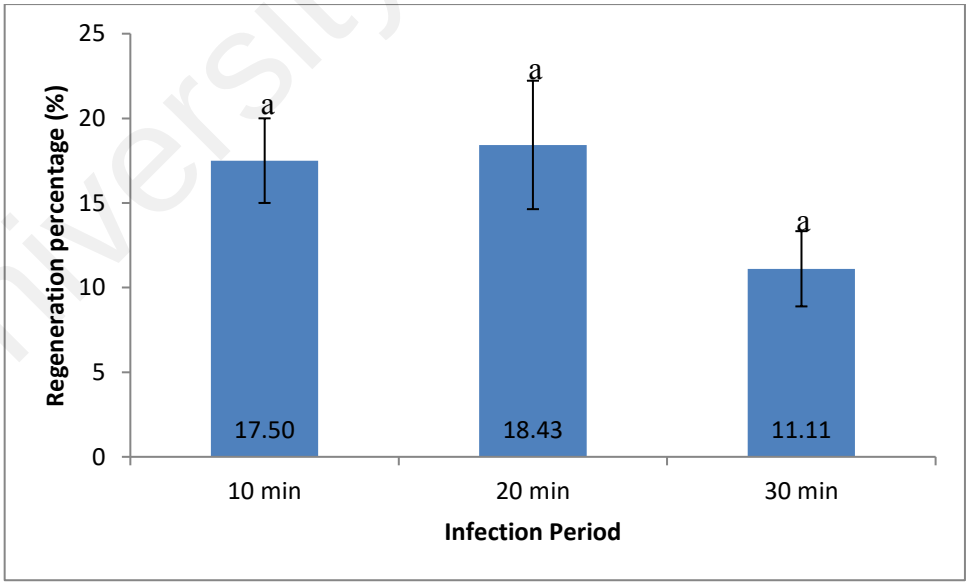


Figure 4.37: Regeneration percentage of *Mucuna bracteata* cotyledonary nodes after infected for different periods with *Agrobacterium tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130. The bars indicate ± standard errors. Mean followed by the same letter are not statistically significant according to Duncan's multiple-range analysis at $p < 0.05$.

4.7.2.2 Molecular analysis of putative *Mucuna bracteata* transformant

(a) ***Crude genomic DNA extraction***

Crude genomic DNA was extracted from the regenerated shoots (Section 3.2.2) and analyzed through gel electrophoresis (Section 3.2.7) as shown in Figure 4.38.

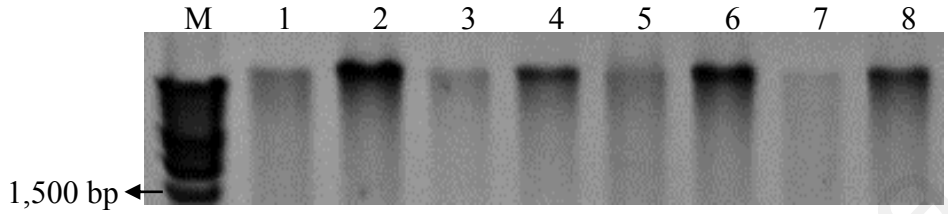


Figure 4.38: Representative image for electrophoretic separation of crude genomic DNA extracted from regenerated shoots on MB selection media. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lanes 1-8: regenerated shoots.

(b) ***Internal control PCR analysis***

Since crude genomic DNA was used as template, internal control PCR analysis using SSR primer (Section 3.7.3.5) was performed to ensure that PCR was not inhibited due to the presence of any unknown PCR inhibitor in the crude genomic DNA. CBT03 primer pair was used in the internal control PCR analysis as shown in Figure 4.39. Two types of positive controls were used for the internal control PCR analysis; genomic DNA of non-transgenic *M. bracteata* extracted using either DNeasy Plant Mini Kit (QIAGEN, USA) (Section 3.2.1) or Edward method (Section 3.2.2) (Section 3.7.3.5). Crude genomic DNA that failed to yield any bands was discarded from further PCR analysis. Crude genomic DNA that yielded similar bands as the positive control was used as template in transgene verification PCR analysis.

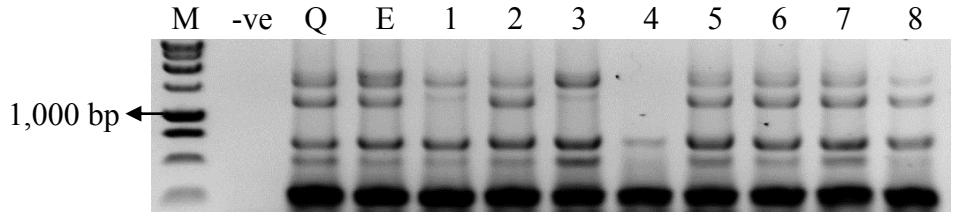


Figure 4.39: Representative image of internal control PCR analysis using CBT03 primer pair. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane –ve: PCR negative control using sdH_2O as DNA template; Lane Q: PCR positive control using genomic DNA of non-transgenic *M. bracteata* extracted using DNeasy Plant Mini Kit; Lane E: PCR positive control using genomic DNA of non-transgenic *M. bracteata* extracted using Edward method; Lanes 1-8: regenerated shoots.

(c) Transgene presence verification PCR analysis

PCR analysis using TRAnpt primer pair was conducted to verify transgene presence in the regenerated shoots (Section 3.7.3.5). Two types of negative controls were used; sdH_2O and genomic DNA of non-transgenic *M. bracteata* extracted using DNeasy Plant Mini Kit (QIAGEN, USA) (Section 3.2.1). Primary PCR was conducted using crude genomic DNA (Section 3.2.2) as DNA template (Section 3.2.6). PCR products from the primary PCR were then used as template in secondary PCR. Regenerated shoot with the presence of 968 bp band in the secondary PCR was considered as putative transformant (Figure 4.40).

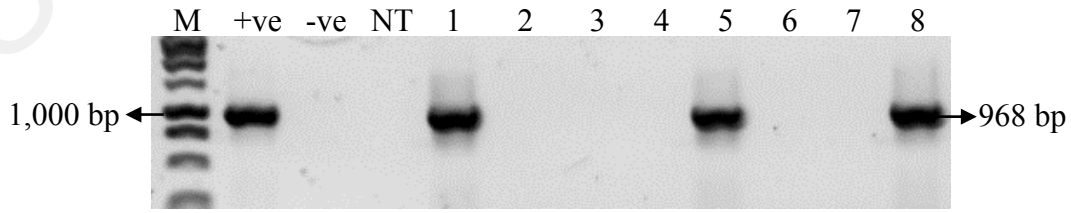


Figure 4.40: Representative image of transgene presence verification PCR analysis using TRAnpt primer pair. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane +ve: PCR positive control using plasmid pTRAkcHcLcTg130; Lane –ve: PCR negative control using sdH_2O as DNA template; Lane NT: genomic DNA of non-transgenic *M. bracteata* as DNA template; Lanes 1-8: regenerated shoots.

The percentage of putative transformant after different infection period is presented in Figure 4.41. Overall, the highest percentage of putative transformant was recorded in shoots regenerated on MB selection media after 20 min of infection period (51.92%). This was significantly higher compared to only 19.05% and 30.95% that were recorded for shoots regenerated on MB selection media after 10 min and 30 min of infection period, respectively.

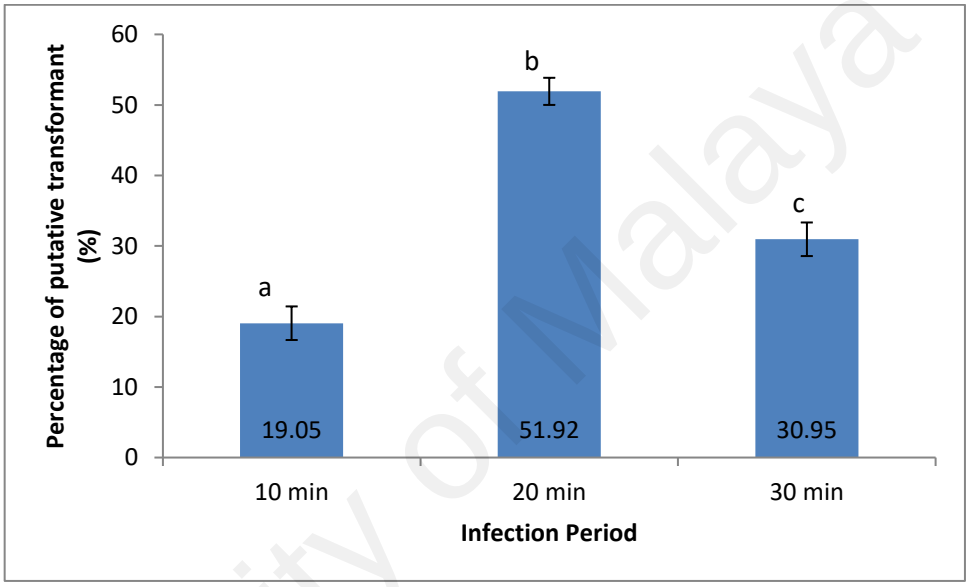


Figure 4.41: Percentage of putative transformants in regenerants of *Mucuna bracteata* cotyledonary nodes after infection for different periods with *Agrobacterium tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130. The bars indicate ± standard errors. Mean followed by the same letter are not statistically significant according to Duncan's multiple-range analysis at $p< 0.05$.

4.7.3 Co-cultivation period

Cotyledonary node explants were infected by immersing the explants in *Agrobacterium* suspension for 20 min (Section 3.7.3.2) and co-cultivated for 2, 4 or 6 days (Section 3.7.3.3). The number of shoots generated after one month of culture on MB selection media (Section 3.7.3.4) was recorded (Section 4.7.3.1).

4.7.3.1 Regeneration percentage on MB selection media

After one month of culture on MB selection media, explants that were co-cultivated for 6 days recorded the highest regeneration percentage (46.53%) compared to 4 days (29.19%) and 2 days (14.96%) of co-cultivation as presented in Figure 4.42. Tissue samples were taken from either the leaves or the shoot tips of regenerants for crude genomic DNA extraction (Section 3.2.2) and PCR analysis (Section 3.7.3.5).

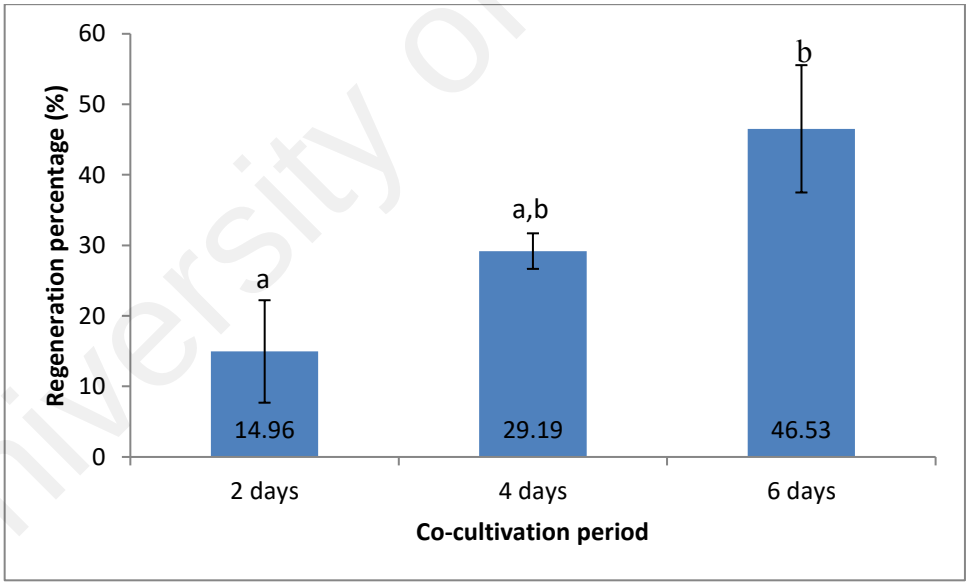


Figure 4.42: Regeneration percentage of *Mucuna bracteata* cotyledonary nodes after co-cultivation for different periods with *Agrobacterium tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130. The bars indicate ± standard errors. Mean followed by the same letter are not statistically significant according to Duncan's multiple-range analysis at $p < 0.05$.

4.7.3.2 Molecular analysis of putative *Mucuna bracteata* transformant

(a) ***Crude genomic DNA extraction***

Crude genomic DNA was extracted from the regenerated shoots (Section 3.2.2) and analyzed through gel electrophoresis (Section 3.2.7) as shown in Figure 4.43.

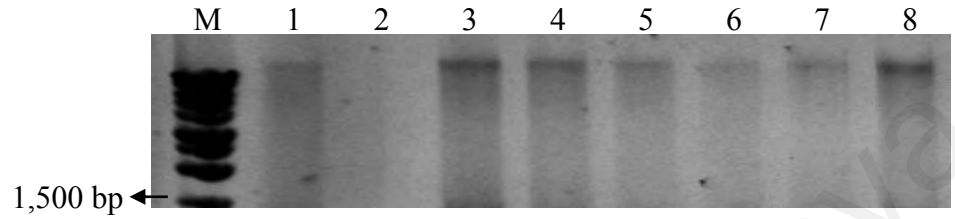


Figure 4.43: Representative image for electrophoretic separation of crude genomic DNA extracted from regenerated shoots on MB selection media. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lanes 1-8: regenerated shoots.

(b) ***Internal control PCR analysis***

Since crude genomic DNA was used as template, internal control PCR analysis using SSR primer (Section 3.7.3.5) was performed to ensure that PCR was not inhibited due to the presence of any unknown PCR inhibitor in the crude genomic DNA. CBT03 primer pair was used in the internal control PCR analysis as shown in Figure 4.44. Two types of positive controls were used for the internal control PCR analysis; genomic DNA of non-transgenic *M. bracteata* extracted using either DNeasy Plant Mini Kit (QIAGEN, USA) (Section 3.2.1) or Edward method (Section 3.2.2) (Section 3.7.3.5). Crude genomic DNA that failed to yield any bands was discarded from further PCR analysis. Crude genomic DNA that yielded similar bands as the positive control was used as template in transgene verification PCR analysis.

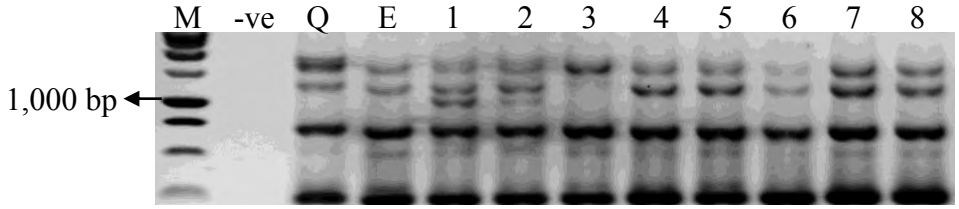


Figure 4.44: Representative image of internal control PCR analysis using CBT03 primer pair. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane –ve: PCR negative control using sdH_2O as DNA template; Lane Q: PCR positive control using genomic DNA of non-transgenic *M. bracteata* extracted using DNeasy Plant Mini Kit; Lane E: PCR positive control using genomic DNA of non-transgenic *M. bracteata* extracted using Edward method; Lanes 1-8: regenerated shoots.

(c) Transgene presence verification PCR analysis

PCR analysis using TRAnpt primer pair was conducted to verify transgene presence in the regenerated shoots (Section 3.7.3.5). Two types of negative controls were used; sdH_2O and genomic DNA of non-transgenic *M. bracteata* extracted using DNeasy Plant Mini Kit (QIAGEN, USA) (Section 3.2.1). Primary PCR was conducted using crude genomic DNA (Section 3.2.2) as DNA template (Section 3.2.6). PCR products from the primary PCR were then used as template in secondary PCR. Regenerated shoot with the presence of 968 bp band in the secondary PCR was considered as putative transformant (Figure 4.45).

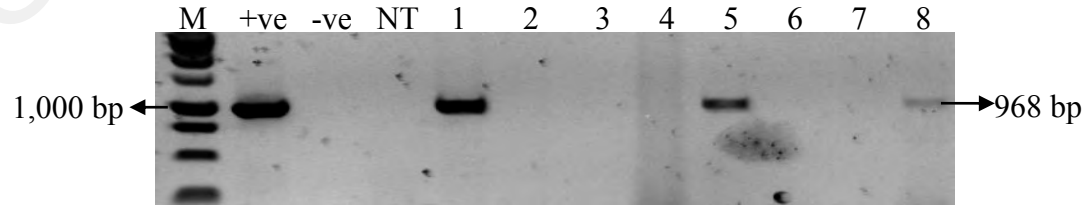


Figure 4.45: Representative image of transgene presence verification PCR analysis using TRAnpt primer pair. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane +ve: PCR positive control using plasmid pTRAkcHcLcTg130; Lane –ve: PCR negative control using sdH_2O as DNA template; Lane NT: genomic DNA of non-transgenic *M. bracteata* as DNA template; Lanes 1-8: regenerated shoots.

The percentage of putative transformant after different co-cultivation periods is presented in Figure 4.46. Overall, the highest percentage of putative transformants was recorded in shoots regenerated on MB selection media after 6 days co-cultivation (31.62%). This was significantly higher compared to only 8.60% and 0.00% that were recorded for shoots regenerated on MB selection media after 4 days and 2 days co-cultivation, respectively.

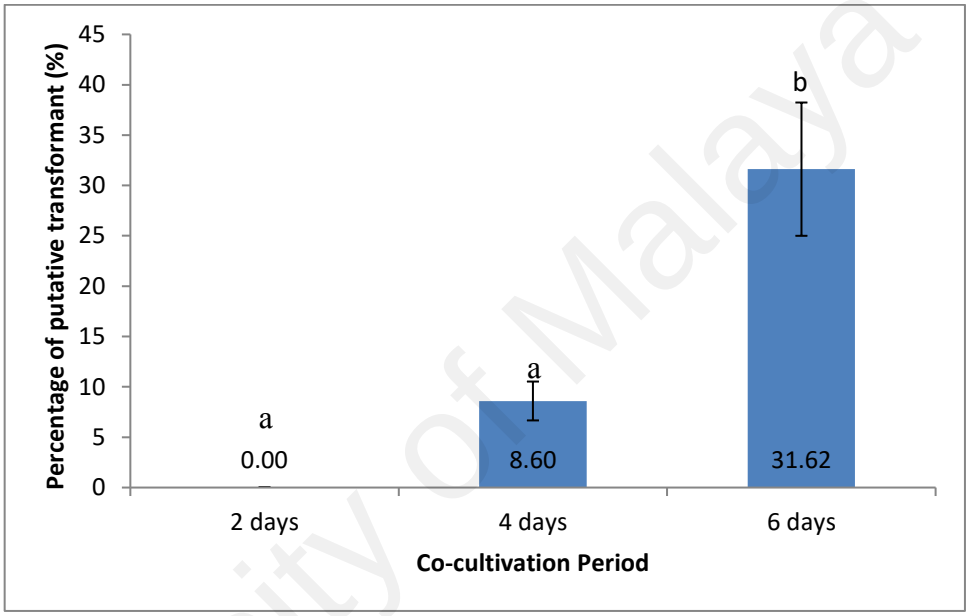


Figure 4.46: Percentage of putative transformants in regenerants of *Mucuna bracteata* cotyledonary nodes after co-cultivation for different periods with *Agrobacterium tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130. The bars indicate ± standard errors. Mean followed by the same letter are not statistically significant according to Duncan's multiple-range analysis at $p< 0.05$.

4.7.4 Maintenance of putative transformants

Based on the results of transgene presence verification PCR analysis from both infection (Section 4.7.2.2) and co-cultivation (Section 4.7.3.2) optimization studies, non-transformed shoots were discarded whereas putative transformed shoots were transferred onto MB selection media with lowered antibiotic concentration (25 µg/mL of G418 disulphate) (Appendix D6) in growing condition as described in Section 3.3.4 to encourage faster growth. The shoots were subcultured onto fresh MB selection media (Appendix D6) every month. However, even though the shoots remained green in color, the shoots growth was completely arrested and no leaves were formed even after five months of culture.

Figure 4.47: Representative images of *Mucuna bracteata* shoots generated from cotyledonary explants transformation with *Agrobacterium tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130. The shoots were maintained on MB selection media with lowered concentration of antibiotic to encourage faster growth. Bar = 1 cm.

4.8 Stable expression of anti-toxoplasma IgG in *Nicotiana tabacum* cv. SR1

4.8.1 Generation of T_0 transgenic lines

Leaf discs of *N. tabacum* cv. SR1 (Section 3.7.4.1) were infected (Section 3.7.4.2) and co-cultivated (Section 3.7.4.3) with *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 (Section 3.7.1) prior to culture on NT selection media (Section 3.7.4.4). A control was setup by culturing non-transformed leaf discs of *N. tabacum* cv. SR1 (Section 3.7.4.1) on NT selection media without the supplementation of kanamycin. After a month of culture, every shoot that was generated from the transformed and non-transformed leaf discs were designated as individual T_0 transgenic and non-transgenic lines, respectively. Genomic DNA was extracted from a leaf of every T_0 transgenic line and three randomly selected T_0 non-transgenic lines (Section 3.7.4.5). The genomic DNA was then used as DNA template for transgene presence verification with TRAnpt primer (Section 3.7.4.5). The results are presented in Figure 4.48.

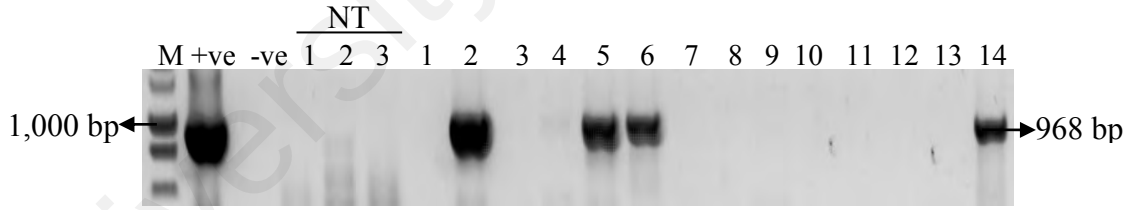


Figure 4.48: Transgene presence verification PCR analysis using TRAnpt primer pair in T_0 transgenic lines. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane +ve: PCR positive control using plasmid pTRAkcHcLcTg130; Lane –ve: PCR negative control using sdH_2O as DNA template; Lane NT: T_0 non-transgenic lines; Lanes 1-14: T_0 transgenic lines.

The PCR analysis showed only four T_0 transgenic lines contained the expected 968 bp band. These four T_0 transgenic lines were considered as true transformants (Figure 4.48). No band was observed from the T_0 non-transgenic lines, confirming that the plants were non-transgenic. The true transformants of T_0 transgenic lines were transferred onto TRM media for root induction (Section 3.7.4.6). The T_0 non-transgenic lines were also transferred onto TRM media for root induction but without the supplementation of kanamycin.

4.8.2 Acclimatization of T_0 transgenic lines

After a month of culture on TRM media for root induction, all the lines generated root excluding NT14 T_0 transgenic line (Figure 4.49). The NT14 T_0 transgenic line remained short and did not form any root structure (Figure 4.49D).

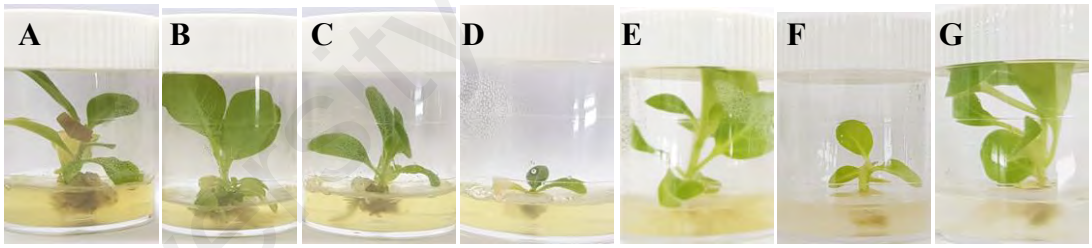


Figure 4.49: Root induction of *Nicotiana tabacum* cv. SR1 T_0 lines. A: NT2 T_0 transgenic line; B: NT5 T_0 transgenic line; C: NT6 T_0 transgenic line; D: NT14 T_0 transgenic line; E-F: T_0 non-transgenic lines.

Excluding NT14 T_0 transgenic line, all of the T_0 transgenic and non-transgenic lines with root formation were acclimatized as described in Section 3.3.5. However, NT2 T_0 transgenic line did not survive acclimatization. T_0 lines that were successfully acclimatized (Figure 4.50) were maintained until flowering for self-crossing (Section 3.7.4.7). Seeds from dried seed pod were collected and designated as T_1 generation.

Figure 4.50: Acclimatized *Nicotiana tabacum* cv. SR1 T_0 lines. Left panel: T_0 transgenic line; Right panel: T_0 non-transgenic lines.

4.8.3 Germination of T_1 generation

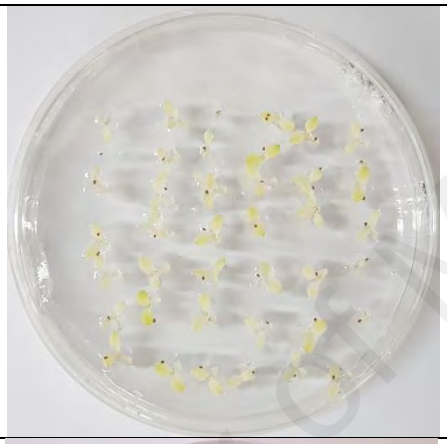
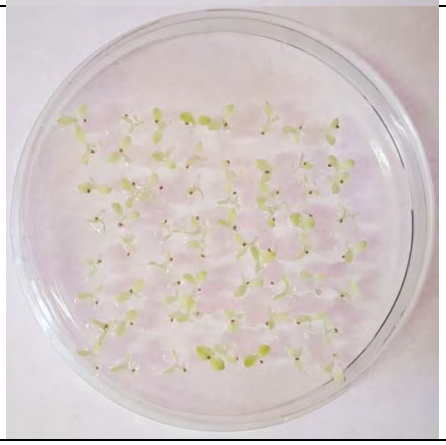
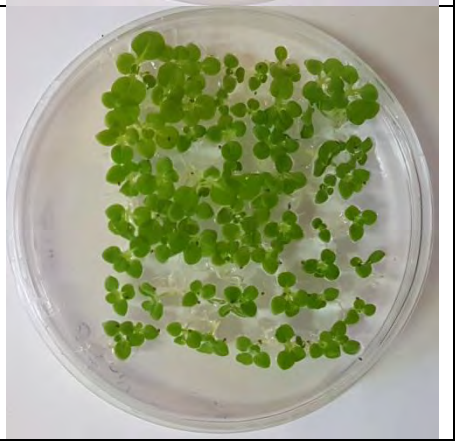
Seeds of T_1 generation were cultured on NT selection media with and without the supplementation of kanamycin (Section 3.7.4.8). Two types of phenotypes were observed in the germinated seedlings; healthy germination (bright green color cotyledon with root formation) and abnormal germination (pale green color cotyledon without root formation). The percentages of seeds with healthy germination are presented in Table 4.10.

Contrary to expectations, only seeds of NT5 T_1 transgenic line healthily germinated (93%) on NT selection media with kanamycin (Table 4.10) (Table 4.11). Similar to non-transgenic seeds, seeds of NT6 T_1 transgenic line germinated abnormally on NT selection media (Table 4.10) (Table 4.11). However, non-transgenic seeds and NT6 T_1 transgenic line seeds germinated healthily on NT selection media without kanamycin (Table 4.11). The results suggested that the transgene was inherited to the T_1 generation of NT5 transgenic line only and not NT6 transgenic line. Thus, only seedlings of NT5 T_1 transgenic line were selected for acclimatization (Section 3.3.5) (Section 3.7.4.8).

Table 4.10: Healthy germination percentage of T_1 seeds on NT selection media with and without kanamycin

	NT selection media	
	with kanamycin	without kanamycin
Non-transgenic	0.00 %	99.2 ± 0.78 %
NT5 transgenic line	93.0 ± 2.89 %	100 %
NT6 transgenic line	0.00 %	99.2 ± 0.78 %

Table 4.11: T_1 seeds germination of transgenic and non-transgenic line.

	NT selection media with kanamycin	NT selection media without kanamycin
Non-transgenic		
NT5 transgenic line		
NT6 transgenic line		

4.8.4 Transgene verification in T_1 generation

Two-week old seedlings of NT5 T_1 transgenic line were randomly selected to be acclimatized (Section 3.3.5) (Section 3.7.4.8). Genomic DNA was extracted from the sixth leaf of 40 day-old NT5 T_1 plants and used as DNA template for transgene presence verification using TRAnpt primer (Section 3.7.4.5). The PCR results confirmed the presence of transgene in the tobacco genome that was stably inherited into the T_1 generation of NT5 transgenic line (Figure 4.51).

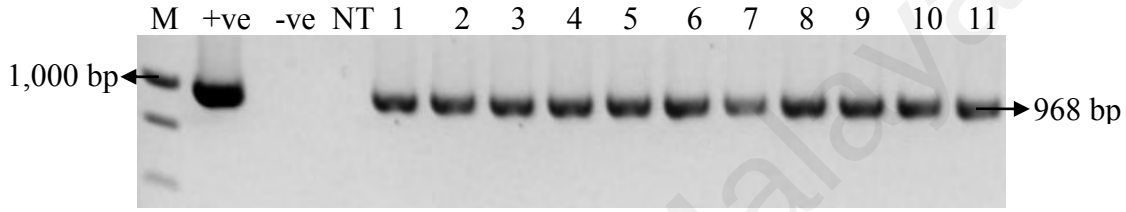


Figure 4.51: Representative image for transgene presence verification PCR analysis using TRAnpt primer pair. Lane M: Plus™ 1 kb DNA Ladder (EURx, Poland); Lane +ve: PCR positive control using plasmid pTRAkcHcLcTg130; Lane –ve: PCR negative control using sdH_2O as DNA template; Lane NT: genomic DNA of non-transgenic *M. bracteata* as DNA template; Lanes 1-11: T1 plants of NT5 transgenic line.

4.8.5 Physiological observation of T1 generation

In addition to the number of leaves and the stem height of each plant at 40-day old (Section 3.7.4.9), the number of days the plants took to flower were also recorded (Section 3.7.4.10). The results are presented in Figure 4.52 and summarized in Table 4.12. At 40-day old, the average number of leaves and stem height of T_1 plants of NT5 transgenic line was 9.62 ± 0.32 leaves and 20.7 ± 1.42 cm, respectively (Table 4.12). This was not significantly different from that of non-transgenic plants (10.2 ± 0.80 leaves; 22.3 ± 1.69 cm) (Table 4.12). However, the average number of days to flower was significantly different between non-transgenic (70 ± 5.11 days) and transgenic (56.81 ± 1.75 days) plants.

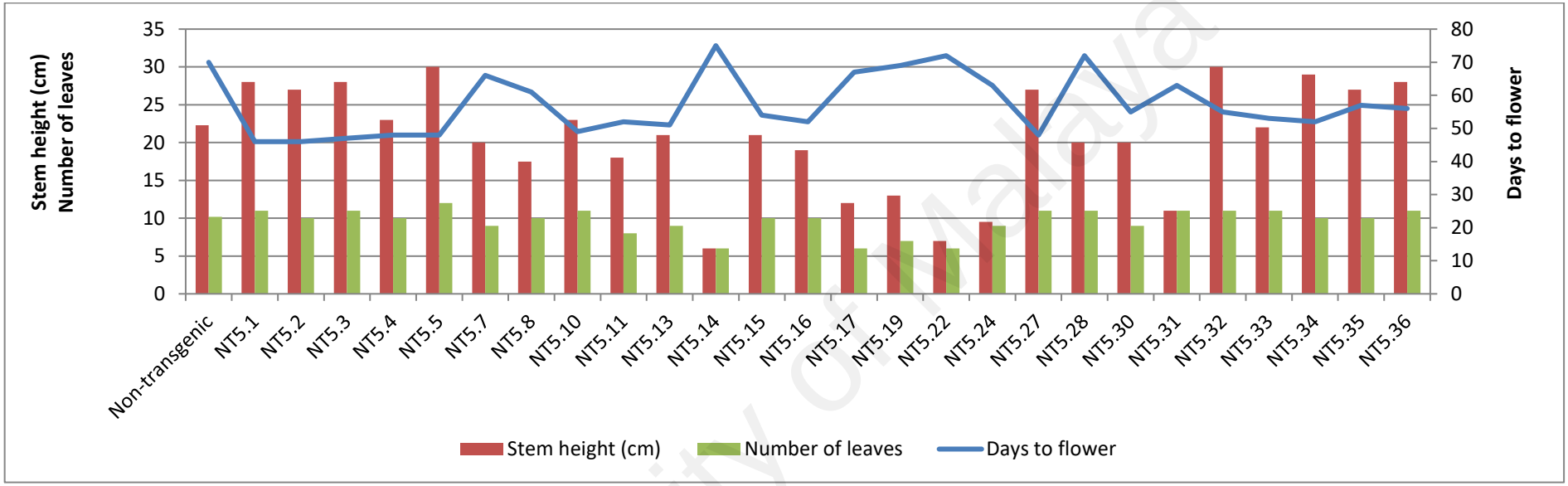


Figure 4.52: Physiological observation of T_1 generation of NT5 transgenic line. The number of leaves and stem height at 40-day old and the number of days to flower for each non-transgenic plant and T_1 plants of NT5 transgenic line of NT5 plant were recorded.

Table 4.12: Summary of physiological observation of T_1 generation of NT5 transgenic line

Phenotype	Average value		Highest		Lowest	
	Non-transgenic	Transgenic	Value	Line	Value	Line
Stem height (cm)	22.3 ± 1.69	20.7 ± 1.42	30.0	NT5.5	6.0	NT5.14
Number of leaves	10.2 ± 0.80	9.62 ± 0.34	12	NT5.5	6	NT5.14
Days to flower	70 ± 5.11	56.81 ± 1.75	75	NT5.14	46	NT5.1 and NT5.2

4.8.6 Anti-toxoplasma IgG concentration in selected T1 transgenic lines

Anti-toxoplasma IgG from the sixth leaf of 40-day old T_1 plants of NT5 transgenic line were extracted using NEB and quantitated (Section 3.7.4.9). The results are presented in Figure 4.53.

The average concentration of anti-toxoplasma IgG in the T_1 plants of NT5 transgenic line was 112.45 ± 10.01 µg/g fresh weight leaf. The highest concentration was observed in NT5.31 plant (256.93 µg/g fresh weight leaf) whereas the lowest concentration was observed in NT5.7 plant (31.47 µg/g fresh weight leaf). No anti-toxoplasma IgG was extracted from non-transgenic plant.

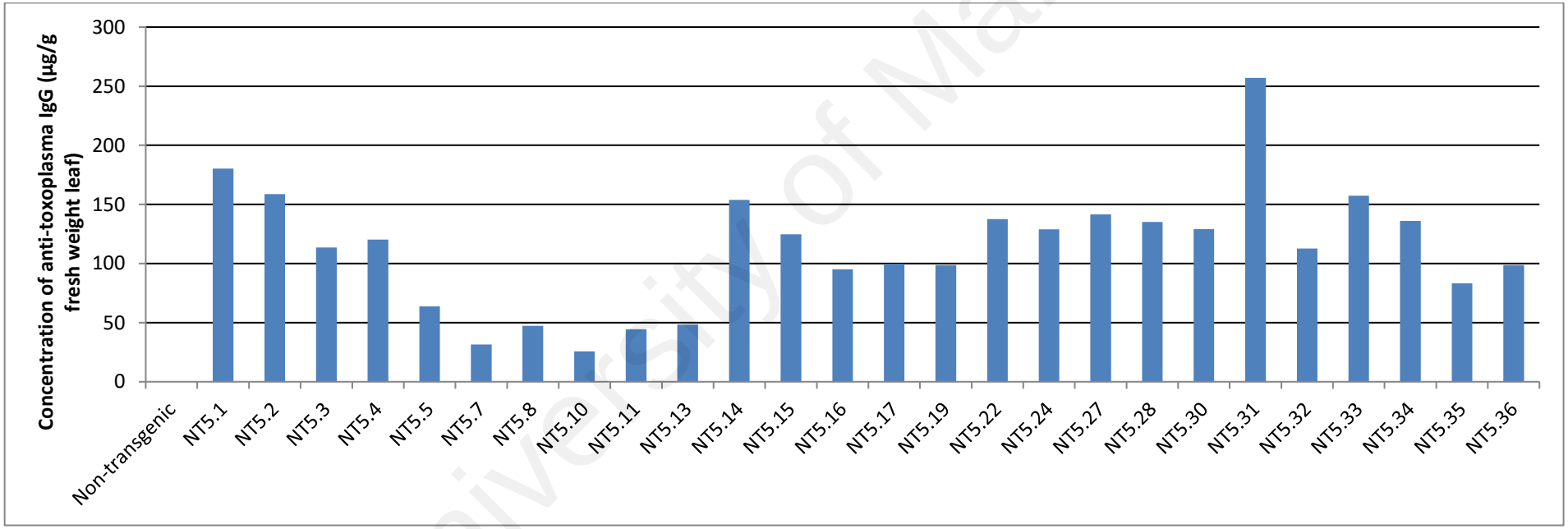


Figure 4.53: Concentration of anti-toxoplasma IgG from the sixth leaf of 40-day old T_1 plants of NT5 transgenic line.

4.8.7 Western blot analysis

Anti-toxoplasma IgG that was extracted and purified (Section 3.4.1 – Section 3.4.6) from T_1 plants of NT5 transgenic line (Section 3.7.4.9) were analyzed via Western blot using anti-human IgG Fab fragment antibody (Section 3.4.7). A band with an estimated molecular mass of ~58 kDa was detected in all of the NT5 transgenic plants (Figure 4.54). As expected, no band was detected from non-transgenic plant.

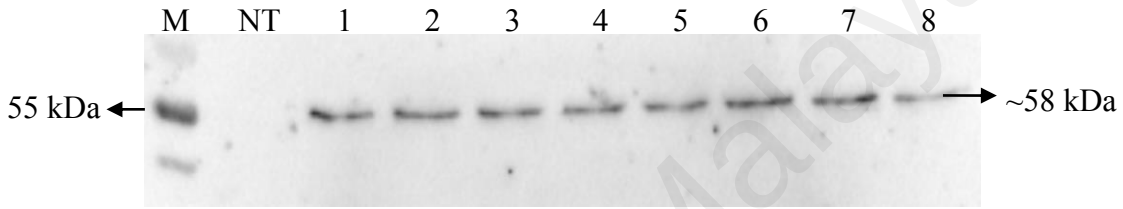


Figure 4.54: Representative image of Western blot analysis of purified anti-toxoplasma IgG from the sixth leaf of 40-day old T_1 plants of NT5 transgenic line. M: EZRun-Prestained Rec Protein Ladder; NT: non-transgenic *Nicotiana tabacum* cv. SR1; 1-8: T_1 plants of NT5 transgenic line.

4.8.8 ELISA

The antigen binding property of anti-toxoplasma IgG extracted and purified (Section 3.4.1 – Section 3.4.6) from T_1 plants of NT5 transgenic line (Section 3.7.4.9) were analyzed via ELISA (Section 3.4.8). Detection of biological activity at an average value of 3.87 ± 0.08 U/mL among all T_1 plants of NT5 transgenic line (Figure 4.55) indicated that the antibody retained its antigen binding properties. The highest activity was recorded by NT5.11 (4.33 ± 0.08 U/mL) whereas the lowest activity was recorded by NT5.7 (2.80 ± 0.03 U/mL). As expected, no functional biological activity was recorded from non-transgenic plant.

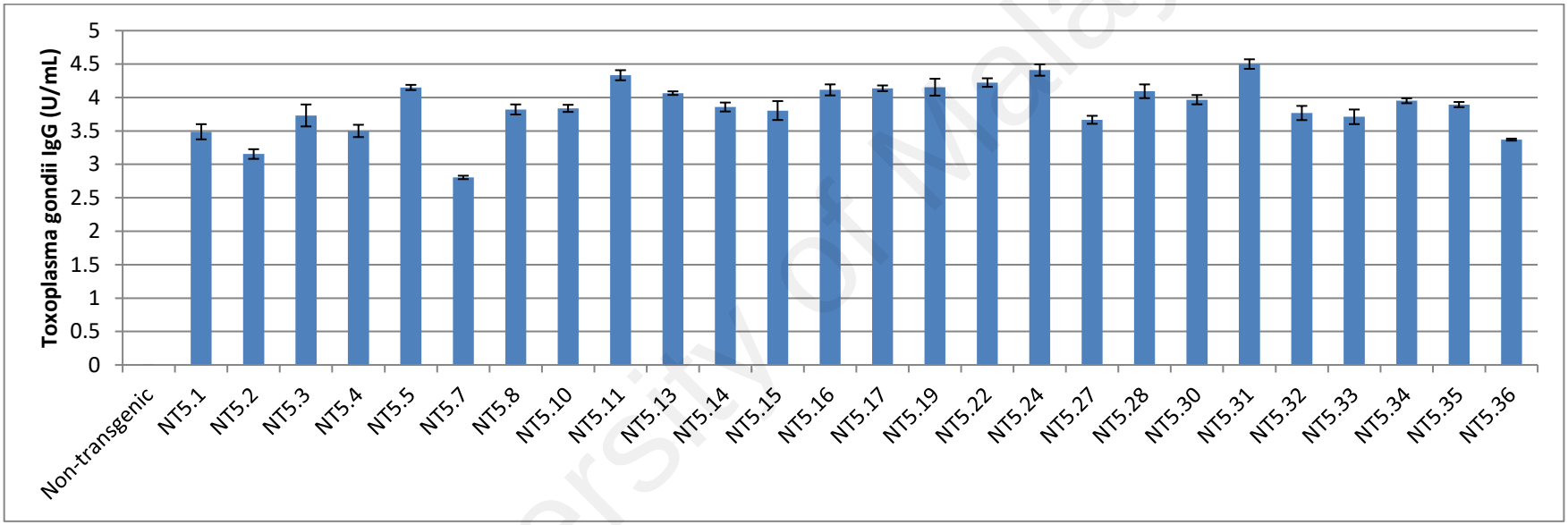


Figure 4.55: ELISA analysis of purified anti-toxoplasma IgG from the sixth leaf of 40-day old T_1 plants of NT5 transgenic line. Analysis was carried out using Human Anti-*Toxoplasma gondii* IgG ELISA kit (Abcam, UK) according to the manufacturer's protocol. The bars indicate mean ± standard errors.

CHAPTER 5: DISCUSSION

5.1 Plasmid pTRAkcHcLcTg130

The plasmid pTRAkcHcLcTg130 encodes for the heavy and light chain components of a mouse-human chimeric anti-toxoplasma IgG (Figure 4.8; Section 4.1). The plasmid backbone contains several features that have been shown to increase heterologous gene expression such as the scaffold attachment region of tobacco RB7 gene, duplicated transcriptional enhancer, codon-optimized murine signal peptide of mAB24, chalcone synthase 5'-untranslated region and endoplasmic reticulum retention signal (Arcalis et al., 2013; Dietz-Pfeilstetter et al., 2016; Mann et al., 2012; Sack et al., 2015; Yamasaki et al., 2018). In addition, the plasmid pTRAkcHcLcTg130 also contains neomycin phosphotransferase as a selectable marker in stable transformation to generate transgenic lines. By having a selectable marker, the plasmid pTRAkcHcLcTg130 can be conveniently used for both transient and stable transformation.

5.2 Development of an *in vitro* propagation protocol for *Mucuna bracteata*

Efficient protocols for seed germination and *in vitro* propagation of *M. bracteata* were established. The entire workflow from seed germination until acclimatization in the greenhouse could be achieved within three months (Figure 5.1). The generated plantlets showed genetic stability after confirmation with SSR analysis (Section 4.4.8). The established protocols were then used in subsequent experiments to establish transient (Section 4.5) and stable (Section 4.7) *Agrobacterium*-mediated transformation procedures for *M. bracteata*. The protocols established in this study could be scalable to mass produce *M. bracteata* plantlets as an alternative source of supply for plantation companies. The protocols may also facilitate future improvement strategies especially for the application of –omic technologies in the study and utilization of this important tropical cover crop.

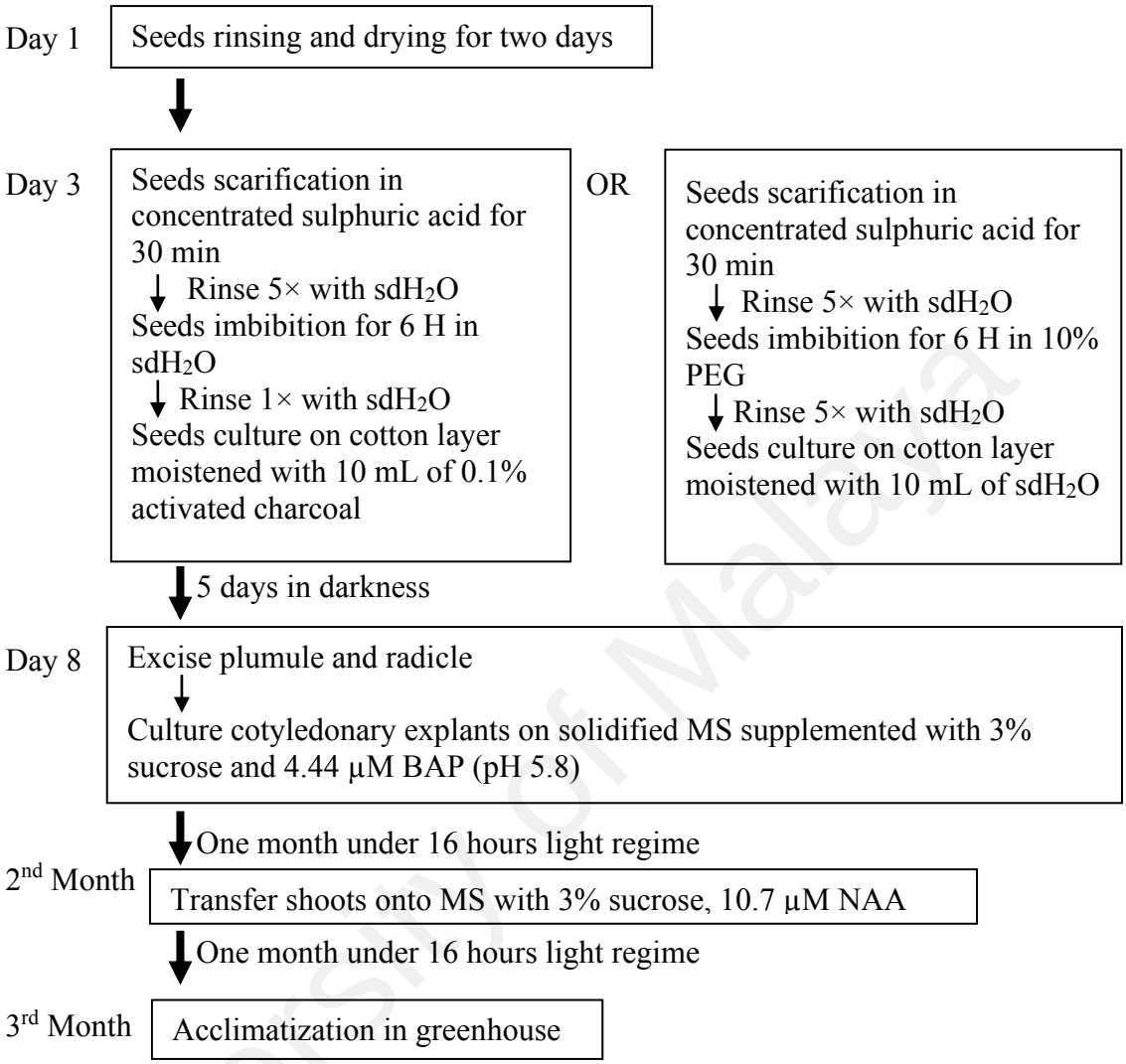


Figure 5.1: General workflow for seeds germination and *in vitro* propagation of *Mucuna bracteata.*

5.2.1 Effect of sulfuric acid treatments on seed germination

Several scarification methods have been widely used to break seed dormancy, such as boiling the seeds in water, clipping the seed coat with a large nail clipper or submerging the seeds in concentrated sulphuric acid (Hermansen et al., 2000). Scarification with sulphuric acid was selected in this study since a large number of seeds can be scarified at one time. The results showed that *M. bracteata* seeds that were submerged in sulphuric acid for 30 min have the highest percentage of germinated seeds compared to the other exposure times (Table 4.1; Section 4.4.1). Similar results were also observed in *Corchorus olitorius* and *Hibiscus trionum* where sulphuric acid scarification for 30 min produced the highest percentage of seed germination (Chachalis et al., 2008; Velempini et al., 2003). Longer scarification period resulted in a lower percentage of germinated seeds. Low scarification efficiency has been reported in seeds exposed to sulphuric acid for a short period of time, while prolonging the exposure period might adversely affect the seed viability (Martín & Guerrero, 2014). For instance, Hermansen et al. (2000) found that seed scarification for more than 90 min decreased the germination rate of *Dimorphandra mollis*. Based on these results, *M. bracteata* seeds were scarified with sulphuric acid for 30 min in subsequent experiments.

5.2.2 Effect of different disinfection protocols on seed germination

Surface sterilization is an important step in establishing *in vitro* plantlets. Clorox is a mild sterilizing agents that has been widely used to disinfect plant material (Srivastava et al., 2010). It kills microorganisms, including bacteria and some viruses, by oxidizing their biological molecules, such as proteins and nucleic acids (Bloomfield et al., 1991; Sawant & Tawar, 2011). Surprisingly, the results showed that there were no significant differences between different surface sterilization treatments that were tested (Table 4.2; Section 4.4.2). A similar finding was also reported by Barampuram et al. (2014), where the use of Clorox® to surface disinfect the cotton seed was not successful. This suggested that disinfection treatment was unnecessary for *M. bracteata* seeds. The immersion of seeds in concentrated sulfuric acid during scarification process might already be sufficient to reduce contamination. Moreover, the use of harsh and toxic chemicals might affect seed development (Barampuram et al., 2014). Therefore, surface sterilization of *M. bracteata* seeds was not performed in subsequent experiments.

5.2.3 Effect of hydropriming conditions on seed germination

Imbibition is important for seed germination as it signals the resumption of embryo growth and the activation of DNA repair mechanisms from dormancy state and metabolic quiescence (Carbonera et al., 2015). Seed priming is a controlled imbibition treatment to initiate seed germination process. The success of seed priming is strongly depended on plant species/genotype and physiology, seed quality as well as the priming method used. There are several priming techniques, namely hydropriming, osmopriming and biopriming. In this study, hydropriming and osmopriming techniques were selected after considering the ease of handling and relatively lower cost compared to other techniques.

In hydropriming, *M. bracteata* seeds that were imbibed for 6 h and cultured on wet cotton roll with 10 mL of sdH_2O produced the highest percentage of germinated seeds (Table 4.3; Section 4.4.3). Increased volumes of sdH_2O to the cotton roll and longer hydropriming period were shown to negatively affect the seed germination. A similar finding has also been reported by Eskandari (2013). This might be attributed to oxygen deprivation where gas diffusion was heavily constrained due to excessive hydration. Seed germination was often characterized with marked increased in oxygen uptake (Weitbrecht et al., 2011). Thus, oxygen availability was crucial. The stress caused by low oxygen availability may induce rapid restriction of metabolism that could affect seed germination (Geigenberger, 2003).

Seed vigor is one of the important aspects in seed development. Previous reports demonstrated a strong correlation between seed vigor with field emergence and performance (Egli & Rucker, 2012; Singh et al., 2014; ur Rehman et al., 2014). Seed vigor has been shown to be affected by the ratio of sucrose/raffinose family oligosaccharides in leguminous *Medicago truncatula* (Vandecasteele et al., 2011) and exogenous application of folic acid and ascorbic acid in *Pisum sativum* (Burguieres et al., 2007). Considering the importance of seed vigor during seedling development, imbibition period of 6 h followed by culturing on wet cotton roll with 10 mL of sdH_2O was regarded as optimal condition for germinating seeds of *M. bracteata*.

5.2.4 Effect of osmopriming and antioxidant treatments on seed germination

Osmopriming protects seeds from oxidative damage caused by reactive oxygen species (ROS) and accelerates seed germination process (Paparella et al., 2015). One of the commonly used osmopriming agents was PEG. Numerous studies have reported that PEG was able to enhance seed germination in many plant species (Bittencourt et al., 2005; Tobe et al., 2000; Yasari et al., 2013). However, osmopriming using PEG is not economically viable as it is expensive and difficult to be removed from the mixture.

Since excessive accumulation of phenolic compounds could adversely affect the seed development (Chon et al., 2002), some studies applied exogenous ascorbic acid or activated charcoal to scavenge ROS or absorb phenolic compounds during seed hydration and germination phases (Mohammadi et al., 2014). Given that phenolic compounds are a major antioxidant constituent in *Mucuna* species (Sridhar & Bhat, 2007; Surveswaran et al., 2007), hydropriming was applied together with 2.27 mM ascorbic acid or 0.1% (w/v) activated charcoal to promote the seed germination. The data revealed that hydropriming treatment with germination on 0.1% (w/v) activated charcoal resulted in comparable seed germination percentage and highest seed vigor index compared to osmopriming or hydropriming treatment alone (Table 4.4; Section 4.4.3). These results demonstrated that the benefits of osmopriming treatment could be offset in hydropriming treatment by culturing the seeds on wet cotton layer supplemented with activated charcoal.

5.2.5 Effect of culture methods and photoperiods on seed germination

The results showed that the percentage of germinated seeds was higher in the dark condition compared to under light exposure (Table 4.5; Section 4.4.4). Earlier studies have reported that seeds with hard seed coat usually have light-independent germination (Chauhan & Johnson, 2008; Huang et al., 2004). There was no difference between culturing the seeds on either MS media or wet cotton roll. Since culturing on wet cotton roll was more economical and feasible compared to MS media, *M. bracteata* seeds were germinated in the dark condition on wet cotton roll in subsequent experiments.

5.2.6 Effect of different types and concentrations of cytokinins on shoot induction and multiplication

In general, cotyledonary nodes were found to be more effective than nodal segments and leaf discs for shoot initiation (Table 4.6; Section 4.4.5). This was in agreement with the study carried out by Husain et al. (2008), where cotyledonary nodes of leguminous tree (*Pterocarpus marsupium* Roxb.) were suitable explants for shoot induction and multiplication. This might be due to the proliferating nature of pre-existing meristems at cotyledonary nodes compared to nodal segments (Distabanjong & Geneve, 1997).

The results also demonstrated that supplementation of cytokinin at low concentration helped to induce higher number of shoots as indicated by previous studies (Al-Bahrany, 2002; Chand & Singh, 2004; Husain et al., 2008). Several studies have shown that BAP was more effective than kinetin for shoot induction (Grzegorczyk-Karolak et al., 2015; Wei et al., 2015; Zaheer & Giri, 2015). Kinetin is known to promote shoot elongation while BAP enhances bud proliferation and multiplication (Diallo et al., 2008). Since the aim was to develop a suitable plant host system for genetic transformation, cotyledonary nodes were selected as explants for shoot induction and multiplication on MS media supplemented with 4.44 μM BAP.

5.2.7 Effect of different types and concentrations of auxins on root induction

Supplementation of MS media with 10.7 µM NAA resulted in the highest percentage of rooting response in cotyledonary node-derived shoots (Table 4.7; Section 4.4.6). NAA is a synthetic auxin that has been widely used to induce roots for many plant species (Al-Bahrany, 2002; Tan et al., 2011; Wei et al., 2015). It has been found to be more effective than IBA in inducing roots for plant species, such as *Arbutus andrachne* (Mostafa et al., 2010) and *Vanilla planifolia* (Tan et al., 2011).

5.2.8 Estimation of DNA content and genome size via flow cytometry analysis

Further analysis using flow cytometry has allowed the first estimations on the DNA content (1.48 ± 0.01 pg) and genome size (1,448 ± 9 Mb) of *M. bracteata* that provides base line data for future molecular cytogenetic study of *M. bracteata* and molecular characterization of *Mucuna* populations in general (Section 4.4.9). Flow cytometry analysis was often employed to assess the trueness-to-type of regenerants for a variety of species such as *Solanum trilobatum* (Shilpha et al., 2014), *Pinus elliottii* (Nunes et al., 2016) and *Vavilovia formosa* (Ochatt et al., 2016). To date, this is the first report on an estimation of the genome size and DNA content for *M. bracteata.*

5.3 Development of transient expression system for *Mucuna bracteata*

5.3.1 Syringe infiltration versus vacuum-assisted infiltration

Syringe infiltration and vacuum-assisted infiltration are important transient transformation methods that are widely used in many plant genetic engineering studies (Leuzinger et al., 2013). Syringe infiltration is usually used in bench scale infiltration by injecting a small volume of *Agrobacterium* suspension into the intercellular space in leaf. On the other hand, vacuum-assisted infiltration is commonly applied for large scale infiltration (Buyel et al., 2017). In vacuum-assisted infiltration, plant tissue is submerged in *Agrobacterium* suspension, followed by vacuum application to permeate *Agrobacterium* suspension into the intercellular space in leaf. This method has been used for different types of plant tissues such as seeds of pea (Fan et al., 2011), nodal explants of *Withania somnifera* (Sivanandhan et al., 2015), whole plant of *N. benthamiana* (Houdelet et al., 2017) and detached sunflower leaves (Jung et al., 2014).

While syringe infiltration allows versatility in experimental design such as requiring only a small volume of culture to infiltrate several *Agrobacterium* cultures that may harbor different constructs on separate segments of the same leaf (Wroblewski et al., 2005; Wydro et al., 2006), this method appears to be dependent on the type of leaf or plant species. This was in agreement with the unsuccessful attempt to syringe infiltrate the leaves of *M. bracteata* (Section 4.5.1). The leaves of *M. bracteata* are brittle and easily damaged when too much pressure was applied during syringe infiltration. Even with extreme caution when applying pressure, the infiltrated area did not expand beyond the syringe nozzle area (Figure 4.22B; Section 4.5.1). This could be attributed to the leaf structure and architecture (Wroblewski et al., 2005). King et al. (2015) reported similar difficulties for legume soybean leaves. The authors found that agroinfiltration could only be achieved with the combination of sonication, reducing agent and vacuum treatment.

Preliminary test on vacuum infiltration of *M. bracteata* plant with *Agrobacterium* suspension has shown promising results as indicated by the presence of dark green patches (Figure 4.22C; Section 4.5.1). Even though these patches were erratic, vacuum-assisted infiltration was more convenient and feasible method for routine study in *M. bracteata* compared to arduous syringe infiltration as exemplified by extensive application of vacuum-assisted infiltration in several industrial recombinant protein production facilities (Holtz et al., 2015; Nandi et al., 2016).

5.3.2 Effect of trifoliate leaves position on transient expression level of anti-toxoplasma IgG

Five week-old *M. bracteata* plant typically has three sets of trifoliate leaves. The oldest trifoliate leaf was located at the bottom of the stem and the leaves become increasingly younger towards the top (Figure 3.6B; Section 3.6.3). This indirectly allowed us to study the effect of leaf age on transient expression of anti-toxoplasma IgG in *M. bracteata*. Various studies have shown that expression levels of heterologous protein varied according to leaf age and position (Bashandy et al., 2015; Buyel & Fischer, 2012; Sack et al., 2015; Wydro et al., 2006). Since *M. bracteata* starts to branch out after six weeks of planting, five week-old *M. bracteata* was used in this study to avoid incoherent and inconclusive comparisons between individuals (Section 4.5.2).

In this study, the bottom trifoliate leaves of five week-old *M. bracteata* produced ~1.5 fold higher concentration of anti-toxoplasma IgG compared to trifoliate leaves at other positions (Figure 4.23; Section 4.5.2.1). The bottom trifoliate leaf was the first true leaf in *M. bracteata*. Wroblewski et al. (2005) demonstrated that higher level of heterologous protein expression was observed in the first true leaves of lettuce, tomato and Arabidopsis than the leaves produced later. However, tobacco exhibited the highest

transient heterologous protein expression either in intermediate or top leaves where rapid cell expansion with high levels of protein synthesis occurred (Buyel & Fischer, 2012; Sack et al., 2015). It is well established that transient heterologous protein expression pattern was species dependent (Buyel & Fischer, 2012; Sheludko et al., 2007).

Different leaf positions might produce different levels of protein expression due to the general changes in leaf physiology (Wydro et al., 2006). Younger leaves have thin leaf lamina that could impair infiltration efficiency (Sheludko et al., 2007) which led to variable expression levels due to uneven *Agrobacterium* infiltration (Yang et al., 2000). This was corroborated by a study in potato plants conducted by Bhaskar et al. (2009). The authors found that the infiltration efficiency in younger plants (3-4 week-old) was significantly lower compared to older plants (5-6 week-old). Taken together, anti-toxoplasma IgG was extracted only from the bottom trifoliate leaf of five week-old *M. bracteata* plant in subsequent experiments.

5.3.3 Transient expression level of anti-toxoplasma IgG over different periods post infiltration

Concentrations of anti-toxoplasma IgG in the bottom trifoliate leaves of five week-old *M. bracteata* were highest at two days and four days post infiltration (Figure 4.24; Section 4.5.2.2). This finding was similar to other plants such as onion epidermis (Xu et al., 2014), *Catharanthus roseus* (Mortensen et al., 2019), rose petal (Yasmin & Debener, 2010) and tobacco (Zheng et al., 2012) where transient heterologous protein expression decreased significantly after 2-3 days post infiltration.

Diminishing transient expression post infiltration was often associated with an active endogenous RNA silencing process and proteolytic degradation in plant cells (Johansen & Carrington, 2001; Voinnet et al., 2003). A study conducted by Wydro et al. (2006)

has showed that co-expression of RNA silencing suppressor led to continuous increase in the expression of the heterologous protein. Instead of decreasing significantly after 2-3 days post infiltration, transient expression continued to accumulate and achieved highest peak of expression after 6-7 days post infiltration. However, a shortened harvesting time and production cycle could decrease downstream costs for a higher overall annual output (Buyel & Fischer, 2012), indicating the importance of cost-benefit analysis.

Many other recent studies have focused on the proteolytic degradation of plant-made pharmaceutical proteins which remains a major hurdle in plant molecular farming (Donini et al., 2015; Hehle et al., 2011; Jutras et al., 2016; Niemer et al., 2014). Several hundreds of endogenous proteases that may directly or indirectly involve in the proteolysis event *ex planta* or *in planta* have been identified in plants genome (Mandal et al., 2016). Future studies should aim to identify specific proteases that are responsible for the degradation of a given recombinant protein (Mandal et al., 2016; Niemer et al., 2014). These proteases activities may lead to partial or complete hydrolysis of recombinant antibody, thus affecting the final yield of intact recombinant proteins in plant systems (Hehle et al., 2015). Among strategies that have been described to elude unintended proteolysis in plant systems include downregulation of host protease expression (Duwadi et al., 2015; Niemer et al., 2014), the removal of protease-susceptible sites by targeted mutagenesis (Grosse-Holz et al., 2018; Hehle et al., 2016) and co-expression of protease inhibitors (Jutras et al., 2016).

5.3.4 Effect of infiltration with different *A. tumefaciens* strains on transient expression level of anti-toxoplasma IgG

A. tumefaciens strains GV3101, EHA105 and LBA4404 were used in this study to determine their effects on transient heterologous protein expression in *M. bracteata*. The highest concentration of anti-toxoplasma IgG was observed when *M. bracteata* was infiltrated with *A. tumefaciens* strain GV3101 (Figure 4.25; Section 4.5.2.3). Both GV3101 (nopaline type) and EHA105 (succinamopine type) contain C58 chromosomal background, whereas LBA4404 was octopine TiAch5 (Hellens et al., 2000). Deeba et al. (2014) has suggested that *Agrobacterium* strains containing different chromosomal background might affect the efficiency of T-DNA delivery.

Other studies have shown that the effectiveness of an *Agrobacterium* strain to infect and deliver the targeted genes into host plants was not only species but also genotype dependent (Guo et al., 2019; Hisano et al., 2017; Wroblewski et al., 2005; Zottini et al., 2008). For example, EHA105 was more efficient compared with LBA4404 and GV3101 to infect reed (Kim et al., 2013). However, GV3101 produced higher transformation efficiency than EHA105, MP90 and AGL1 in tomato cultivar Micro-Tom (Chetty et al., 2013). Switchgrass transformation was most efficient when AGL1 was used compared with GV3101 and C58 (Chen et al., 2010). In another study, Yasmin and Debener (2010) reported that there were no significant differences in transformation efficiency between GV3101, EHA105, C58C1 and 80.1 infections on various rose genotypes.

The condition of the plant tissue post infiltration was also an important factor to determine the most suitable *Agrobacterium* strain for infection. Unlike most studies, there was no observable necrotic lesion in the infiltrated *M. bracteata* leaves from any of the *Agrobacterium* strains tested. Certain *Agrobacterium* strain resulted in higher mortality rate among the infected explants (Chetty et al., 2013). Transformation of plant tissue using *Agrobacterium* may trigger innate defense response which could lead to necrotic reaction and plant cell death (Gohlke & Deeken, 2014; Pruss et al., 2008). These were often observed in banana transformation where rapid necrotic reaction led to high rate of explants mortality and impeded the overall progress in banana transformation via *Agrobacterium*-mediated approach (Khanna et al., 2007; Zhang et al., 2013).

5.3.5 Effect of different extraction buffers on anti-toxoplasma IgG concentration

Anti-toxoplasma IgG yield was higher when crude protein was extracted using NEB compared to PBS (Figure 4.26; Section 4.5.2.4). Crude protein extraction from *M. bracteata* using PBS often yielded a reddish-brown crude extract that sometimes precipitated after half an hour even when frozen or maintained at 4 ºC. This might be due to the effect of "enzymic browning" where phenolic compounds formed irreversible covalent linkages with proteins, resulting in hydrophobic products that were more susceptible to protein aggregation and precipitation (Charmont et al., 2005; Pierpoint, 2004). In this study, the protein extraction buffer was supplemented with PVPP as phenolic binding agent. PVPP is insoluble and can be easily removed from the extracts by centrifugation. It has been shown that protein quality and quantity were enhanced when PVPP was used during protein extraction from recalcitrant plants (Charmont et al., 2005; Wang et al., 2007; Yao et al., 2006). In addition, water soluble PVP was also incorporated to protect the extracted crude protein from unnecessary reaction with free

phenolic. Indeed, crude protein extraction from *M. bracteata* using NEB produced green protein extract that did not precipitate. These observations suggested that "enzymic browning" in *M. bracteata* crude protein extract could be reduced by incorporating phenolic adsorbents in the extraction buffer to minimize protein-phenolic unspecific binding.

An additional component such as detergent (Triton X-100) was included in NEB to disrupt cellular membranes and maximized protein release into the extraction buffer during protein extraction. Triton X-100 is a mild non-ionic detergent with low tendency to denature proteins and break up protein complexes (Brown & Audet, 2008). Another component that was added into the NEB was glycerol to stabilize the extracted proteins. Glycerol is one of the most widely used polyols and is routinely used in protein refolding and crystallization (Vagenende et al., 2009). Glycerol prevents protein aggregation by inhibiting protein unfolding and stabilizing aggregation-prone intermediates (Vagenende et al., 2009). NEB buffer that was developed for the extraction of crude protein in this study could be used in future proteomic studies of *M. bracteata* or other phenolic-rich plant species.

5.4 Development of stable expression system for *Mucuna bracteata*

5.4.1 Natural regeneration capacity of *M. bracteata* under different antibiotic selection pressure

The gene *nptII* within pTRAkcHcLcTg130 (Figure 4.8; Section 4.1) encodes for neomycin phosphotransferase II which confers resistance towards aminoglycoside antibiotics such as kanamycin, neomycin and G418 disulphate (Fuchs et al., 1993). It is widely exploited as an antibiotic resistance marker gene for selection of transgenic plants. Among all of the aminoglycoside antibiotics, kanamycin is the most commonly used selective agent in various plant species (Chakraborty et al., 2016; Hayta et al., 2018; Itaya et al., 2018; Numata et al., 2016).

In this study, cotyledonary node explants were cultured on MS media supplemented with 4.44 µM of BAP and various concentrations of kanamycin (50-150 µg/mL) or G418 disulphate (12.5-75 µg/mL) to determine the optimal antibiotic concentration for selection and generation of transgenic *M. bracteata* (Section 3.7.2). The results indicated that *M. bracteata* was highly resistant towards kanamycin compared to G418 disulphate (Figure 4.33; Section 4.7.1.1). High tolerance level of legumes towards kanamycin has been previously reported in numerous studies (Igasaki et al., 2000; Jube & Borthakur, 2009; Ko et al., 2011; Xie & Hong, 2002). High concentration of selective agent may affect adversely on the regeneration capability of transgenic explants. Thus, an alternative selective agent with low concentration was preferred. G418 disulphate was often used as an alternative to kanamycin in kanamycin resistant plant species or genotypes (Chong-Pérez et al., 2012; Collado et al., 2016; G. Liu et al., 2019; Yang et al., 2018). In these reported studies, the concentration of G418 disulphate that was required in the selection of transgenic plants was often low but varied between 10 µg/mL to 50 µg/mL. This highlighted the need to determine the optimal concentration of selective agent for the species studied.

The results also demonstrated different sensitivity towards G418 disulphate between shoot regeneration and root induction process in *M. bracteata* (Figure 4.35; Section 4.7.1.2). Similar observations were recorded in *Pyrus ussuriensis* Maxim and *Vigna mungo* L. Hepper where the concentration of kanamycin during shoot regeneration was decreased three-fold (Yang et al., 2017) and seven-fold (Saini et al., 2003), respectively during root induction. In certain protocols, complete removal of selective agent during root induction was necessary to facilitate healthy root growth (An et al., 2014; Collado et al., 2016; Maleki et al., 2018; Mano et al., 2014).

5.4.2 Determination of optimal infection and co-cultivation period

The development of an *Agrobacterium*-mediated transformation requires an optimization of several factors that affect transformation efficiency. Infection and co-cultivation period are among the most important parameters in *Agrobacterium*-mediated transformation since *Agrobacterium* adsorption to plant tissue occurs during these stages (An et al., 2014; Jiang et al., 2015; Kuo et al., 2018). In this study, cotyledonary explants of *M. bracteata* were infected and co-cultivated with *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 for different periods (Section 3.7.3). The results showed that the optimal period was 20 min (Figure 4.41; Section 4.7.2) and 6 days (Figure 4.46; Section 4.7.3) for infection and co-cultivation periods, respectively.

Prolonging or shortening the infection period led to decreased transformation efficiency in *M. bracteata* (Figure 4.41; Section 4.7.2). Similar observations were widely observed in various plant species such as finger millet (Satish et al., 2017), ramie (An et al., 2014), *Populus* (Maheshwari & Kovalchuk, 2016) and mung bean (Yadav et al., 2012). Decreased transformation efficiency as a result of prolonging infection period was often associated with *Agrobacterium* overgrowth and plant tissue necrotic reaction (An et al., 2014).

In contrast to infection period, prolonging co-cultivation period up to six days resulted in higher transformation efficiency in *M. bracteata* (Figure 4.46; Section 4.7.3). Other studies have reported an optimal co-cultivation period of less than five days in various plant species (Jiang et al., 2015; Kuo et al., 2018; Li et al., 2017; Satish et al., 2017; Sivanandhan et al., 2015). Similar to infection period, these studies reported that prolonging co-cultivation period resulted in decreased transformation efficiency due to *Agrobacterium* overgrowth and plant tissue necrotic reaction. However, *Agrobacterium* overgrowth and plant tissue necrotic reaction were not evident in any of the infection and co-cultivation period tested for *M. bracteata* cotyledonary explants transformation. Clearly, the optimal condition for *Agrobacterium*-mediated transformation differed according to plant species and cultivar (Sedaghati et al., 2019).

5.4.3 Regeneration of putative transformant

One of the main obstacles in genetic transformation of various plant species particularly legume is regenerating transformed explants into healthy plantlets (Jaiwal et al., 2001; Ziemienowicz, 2014). This can be observed in pea (*Pisum sativum* L) and faba bean (*Vicia faba* L.) despite their long history of use in plants genetic modification (Ochatt et al., 2018). Similarly, the leguminous *M. bracteata* has demonstrated remarkable recalcitrance to *in vitro* regeneration following *Agrobacterium*-mediated transformation (Section 4.7.4). In this study, numerous attempts to regenerate transgenic *M. bracteata* explants into healthy plantlets have remained unsuccessful. The explants growth remained impeded even after prolonged period of culture on growth media with reduced concentration of selective agent (Section 4.7.4). Because of this, plant tissue material was insufficient for further analysis such as Southern blotting to verify transgene integration into the plants genome and protein extraction to quantitate anti-toxoplasma IgG expression.

It remained unclear whether the inefficient recovery of transgenic plantlet was due to either poor *Agrobacterium* susceptibility or species dependency. Similar observation was reported by Sorrentino et al. (2005) in recovering stable transgenic tobacco cell cultures expressing recombinant human tissue transglutaminase. The authors postulated that accumulation of recombinant protein at high level within cytosol of the cell cultures might have prevented regeneration and growth of the transformed cells. Dugdale et al. (2014) has proposed the use of an inducible expression system that separated plant growth and protein production phases to circumnavigate the inhibitory effect of heterologous protein accumulation to plant development.

In recent years, successful recoveries of transgenic plantlets from other plant species were achieved via various strategies. These include wounding of explants (Sedaghati et al., 2019), supplementation of culture media with ethylene inhibitors (Ashwani et al., 2017; Sgamma et al., 2015), antioxidant compounds (Yang et al., 2016) or thiol compounds (Li et al., 2017; Sivanandhan et al., 2015) and culturing explants on media supplemented with plant growth regulator prior to *Agrobacterium*-mediated transformation (Maheshwari & Kovalchuk, 2016; Sedaghati et al., 2019; Yang et al., 2017). Despite these established protocols, poor reproducibility and inefficiency continue to call for further improvisation and novel approach to recover transgenic plantlets from different plant species.

Using the optimal parameters (infection and co-cultivation period of *Agrobacterium*) determined in this study as the baseline, a combination of some of these reported strategies could be applied in the future recoveries of transgenic *M. bracteata* plantlets. Possibly, an entirely novel approach has to be proposed specifically for *M. bracteata* as it is well established that many protocols in plants research are species/genotype dependent.

5.5 Comparison of anti-toxoplasma IgG expression in different systems

5.5.1 Anti-toxoplasma IgG detection and antigen binding retention analysis

Anti-toxoplasma IgG extracted and purified from *M. bracteata, N. benthamiana* and *N. tabacum* cv. SR1 were analyzed via Western blot using an anti-human IgG Fab fragment antibody (Section 4.5.4) (Section 4.6.2) (Section 4.8.7). A band with an estimated molecular mass of ~58 kDa was detected in all of the plants (Figure 4.28; Figure 4.31; Figure 4.54). However, the estimated molecular mass based on the amino acid sequence of anti-toxoplasma IgG was ~51 kDa. Deviation from the theoretical molecular size could be attributed to glycosylation. Indeed, one of the advantages of using plants as a production platform to produce recombinant pharmaceutical protein is its capability to perform complex post-translational modifications as eukaryotes. Glycosylation is the most common form of modification in eukaryotic cells where at least 50% of human proteins are glycosylated (Webster & Thomas, 2012). N-linked glycosylation pathways in plants are relatively well characterized and share a high degree of homology with other eukaryotic organisms (Webster & Thomas, 2012).

Structural heterogeneity was observed in purified anti-toxoplasma IgG from *M. bracteata* (Figure 4.28; Section 4.5.4), but not from *N. benthamiana* (Figure 4.31; Section 4.6.2) and *N. tabacum* cv. SR1 (Figure 4.54; Section 4.8.7). The presence of an additional band in *M. bracteata* with an estimated molecular mass of ~51 kDa (Figure 4.28; Section 4.5.4), similar to the theoretical molecular mass, suggested the existence of anti-toxoplasma IgG devoid of glycan moiety. It was worthy to note that the ~51 kDa band was less intense compared to the ~58 kDa band (Figure 4.28; Section 4.5.4). This suggested that there was more glycosylated than non-glycosylated anti-toxoplasma IgG in *M. bracteata*. The lack of glycan moiety could be an indication that some recombinant protein in *M. bracteata* experienced incomplete post-translational modification. However, it was suggested that structural heterogeneity resulted

predominantly from degradation rather than partial assembly within cells (Hehle et al., 2015). Proteolytic degradation in plant-derived IgG antibodies was frequently reported (Donini et al., 2015). The glycan moiety could have been cleaved off either *in planta* (during protein synthesis) or *ex planta* (during protein extraction or purification) by proteases that exist in plants (Benchabane et al., 2008). Goulet et al. (2012) reported that different plant species contained diverse types of proteases that were upregulated during agroinfiltration as part of plant immune response against pathogen infection (Clemente et al., 2019). Degradation of plant-derived IgG antibodies due to proteolysis was frequently reported (Donini et al., 2015; Goulet et al., 2012; Hehle et al., 2015; Lallemand et al., 2015; Niemer et al., 2014). Previous study has also shown that residual protease activity could still occur even in highly purified monoclonal antibodies (Gao et al., 2011). Several counteracting strategies such as co-expression of proteases inhibitor (Grosse-Holz et al., 2018) and silencing of proteases gene (Duwadi et al., 2015) have been reported but these strategies rely on a prior knowledge on proteases profile in the species studied. This underlines the importance for future work to better understand the post-translational mechanisms, proteases profile and proteolytic degradation activity in *M. bracteata* as product authenticity and homogeneity are one of the major concerns for plant derived recombinant pharmaceutical protein.

Even though the purified anti-toxoplasma IgG from *M. bracteata* suggested the presence of structural heterogeneity, the purified product retained its antigen binding properties (Figure 4.29; Section 4.5.5) at a comparable level with anti-toxoplasma IgG extracted from *N. benthamiana* (Figure 4.32; Section 4.6.3) and *N. tabacum* cv. SR1 (Figure 4.55; Section 4.8.8) in ELISA.

5.5.2 Comparison of transient expression systems between *M. bracteata* and *N. benthamiana*

In this study, the optimal parameters for transient expression of anti-toxoplasma IgG in *M. bracteata* (Section 4.5.3) were determined. Five week-old *M. bracteata* plant was vacuum-assisted infiltrated (Figure 4.22; Section 4.5.1) with *A. tumefaciens* strain GV3101 (Figure 4.25; Section 4.5.2.3). Subsequently, the bottom trifoliate leaf (Figure 4.23; Section 4.5.2.1) was harvested two days post infiltration (Figure 4.24; Section 4.5.2.2) and crude protein was extracted with NEB buffer (Figure 4.26; Section 4.5.2.4). The concentration of anti-toxoplasma IgG extracted from *M. bracteata* under these optimal transient expression conditions was then compared with *N. benthamiana* which is widely used as a model plant for transient production of plant-derived heterologous protein (Mazalovska et al., 2017; Menassa et al., 2012; Strasser et al., 2008; Sukenik et al., 2018; Wroblewski et al., 2005; Xiong et al., 2018). It has high susceptibility to *Agrobacterium* infection and high leaf to plant biomass ratio (Holtz et al., 2015; Pillay et al., 2016). In this study, the sixth leaf of 40 day-old *N. benthamiana* plant was syringe infiltrated with *A. tumefaciens* strain GV3101 harboring pTRAkcHcLcTg130 according to the established method of Leuzinger et al. (2013).

The results showed that *M. bracteata* consistently produced at least twofold higher concentration of anti-toxoplasma IgG (591.10 ± 52.5 µg/g fresh weight leaf) (Section 4.5.3) compared to *N. benthamiana* (276.61 ± 9.52 µg/g fresh weight leaf) (Section 4.6.1). This antibody yield was also comparatively higher than previous reports using transient based expression system in *N. benthamiana*; 299 ± 35 µg/g fresh weight leaf (Melnik et al., 2018) and 81 ± 17 µg/g fresh weight leaf (Teh et al., 2014b).

It is noteworthy that the parents plasmids, namely pTRAkc-Hc and pTRAkc-Lc (Section 3.1), used in the construction of pTRAkcHcLcTg130 (Section 4.1) in this study were used by Lim et al. (2018) to transiently expressed anti-toxoplasma IgG through co-transformation method in *N. benthamiana*. The authors obtained anti-toxoplasma IgG at concentrations of 33-72 µg/g fresh weight leaf. The higher antibody yield achieved in this study might be due to the usage of antibody tandem constructs pTRAkcHcLcTg130. Co-transformation of separate constructs often yields lower expression level than tandem constructs due to independent integration into the host genome in co-transformation (Heitzer & Zschoernig, 2007).

In terms of cultivation period, the entire process from seeds germination, sowing, cultivation and harvesting post infiltration was shorter for *M. bracteata* compared to *N. benthamiana* (Figure 5.2). *M. bracteata* required a total of 45 days; 8 days for seeds germination, 35 days for growth prior to infiltration and an additional 2 days for post-infiltration period. On the other hand, *N. benthamiana* required a total of 54 days; 8 days for seeds germination, 40 days for growth prior to infiltration and an additional 6 days for post-infiltration period. Shortening cultivation period by nine days could be translated to a huge cost reduction from reduced electricity and water usage in an industrial cultivation setting. Shortened cultivation period also makes it possible to increase crop production cycles (roughly eight production cycles per year for *M. bracteata* compared to roughly only six production cycles per year for *N. benthamiana*) which ultimately increases annual production capacity (Buyel & Fischer, 2012). Further cost reduction was possible with leguminous *M. bracteata* through reduced application of fertilizer compared to *N. benthamiana* (Section 3.3.5) (Figure 5.2).

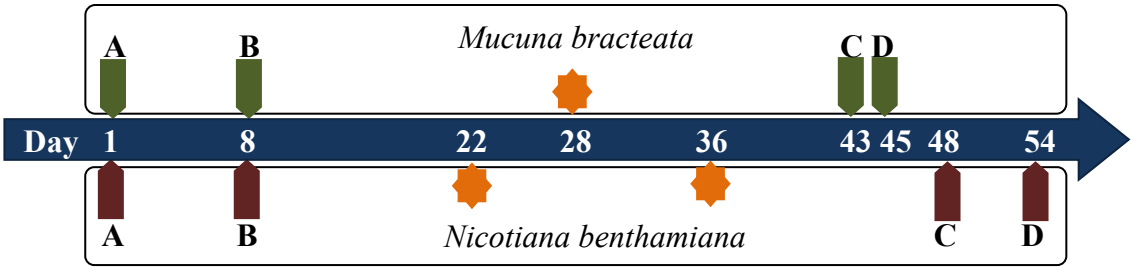


Figure 5.2: Visual timeline of anti-toxoplasma IgG transient expression in *Mucuna bracteata* and *Nicotiana benthamiana*. A: seeds germination; B: seedlings planting; C: *Agrobacterium* infiltration; D: leaves harvesting; : fertilizer application; : *M. bracteata*; : *N. benthamiana.*

5.5.3 Comparison of stable expression systems between *M. bracteata* and *N. tabacum* cv. SR1

This study also attempted to stably express anti-toxoplasma IgG in *M. bracteata* and the model plant *N. tabacum* cv. SR1. As the first genetically modified plant (De Block et al., 1984), *N. tabacum* has been extensively used in various studies that require generation of stable transgenic lines due to its good growth characteristic in glasshouse, high biomass and high number of seeds per plant (Chincinska et al., 2019; Garabagi et al., 2012; Ma et al., 2015; Sack et al., 2015).

A stable transgenic line of *N. tabacum* cv. SR1 expressing anti-toxoplasma IgG in T_1 generation has been successfully generated in this study (Figure 4.53; Section 4.8.6). However, the concentration of anti-toxoplasma IgG varied greatly (31.47 - 256.93 µg/g fresh weight leaf) between selected progenies of T_1 generation even though they were derived from the same T_0 transgenic line. Similar observations were also reported in other studies (Kang et al., 2017; Kirchhoff et al., 2012; Ma et al., 2015; Muthamilselvan et al., 2016). Varying expression levels of recombinant protein were not only confined among individual progenies, but also in *in vitro* regenerants derived from the same

transgenic plants (Kang et al., 2017). It was possible to further increase the yield of recombinant protein in subsequent generations by self-pollinating selected transgenic plants with the highest expression level. However, the yield started to plateau from the fourth generation (T_4) onwards (Sack et al., 2015).

This study has successfully determined the optimal infection (Figure 4.41; Section 4.7.2) and co-cultivation periods (Figure 4.46; Section 4.7.3) for *Agrobacterium*-mediated transformation of *M. bracteata* cotyledonary nodes (Section 5.4.2). Nonetheless, the regenerated transgenic shoots were recalcitrant in growth (Section 4.7.4) (Section 5.4.3). Thus, anti-toxoplasma IgG extraction and quantification could not be conducted due to insufficient plant materials (Section 5.4.3). Therefore, a direct comparison of anti-toxoplasma IgG concentration between stable transgenic lines of *M. bracteata* and *N. tabacum* cv. SR1 could not be performed in this study. If full grown transgenic lines of *M. bracteata* was successfully recovered, differences in antibody yield was anticipated as recently reported (Vafaee & Alizadeh, 2018).

5.5.4 Comparison between transient and stable expression systems

Generation of stable transgenic lines to express recombinant protein is a time-consuming (months to years), resource-intensive and inefficient process. As shown in this study, at least seven months were required to generate stable transgenic lines of *N. tabacum* cv. SR1 expressing anti-toxoplasma IgG (Figure 5.3). Based on the tissue culture protocol developed initially, transgenic shoots from *Agrobacterium*-mediated transformation of *M. bracteata* cotyledonary nodes could be generated within three months; one month each for shoots regeneration, elongation and root induction. However, due to its recalcitrance to regenerate after genetic modification, future works involving optimization of transgenic shoots regeneration are indispensable to obtain a stable transgenic line in *M. bracteata* species (Section 5.4.3) (Section 5.5.3).

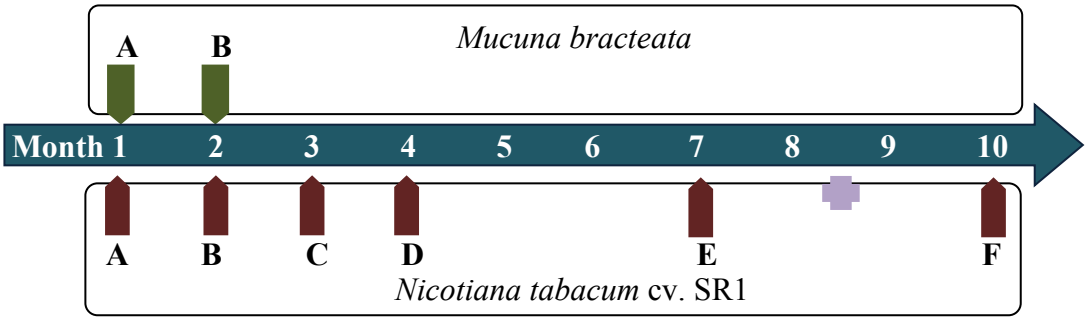


Figure 5.3: Visual timeline required for generating stable transgenic lines expressing anti-toxoplasma IgG in *Mucuna bracteata* and *Nicotiana tabacum* cv. SR1. A: *Agrobacterium*-mediated transformation; B: shoots elongation; C: root induction; D: acclimatization; E: T_1 seeds harvesting and germination; F: T_2 seeds harvesting; : leaves harvesting for anti-toxoplasma IgG extraction; : *M. bracteata*; : *N. tabacum* cv. SR1.

In contrast, recombinant protein production via transient expression system is a rapid, flexible and feasible process (Sainsbury & Lomonossoff, 2014). As discussed previously in Section 5.5.2, anti-toxoplasma IgG could be produced within 45 days in *M. bracteata* and 54 days in *N. benthamiana* (Figure 5.2). Rapid production of critical recombinant protein such as antibody or vaccines is crucial especially in emergency situations such as a pandemic or bioterrorism attack. Several production facilities based on transient expression system, each one with the capacity to grow millions of *N. benthamiana*, have already been built and are currently operational (Holtz et al., 2015; Nandi et al., 2016).

However, in terms of cost-benefit analysis, further cost reduction in recombinant protein production from plants could be made by adopting stable expression system as opposed to transient expression system (Melnik et al., 2018). In transient expression system, bacterial cultures and infiltration media have to be freshly prepared for every production cycle. In contrast, minimal upstream process is involved in stable expression system as leaves could be harvested directly from stable transgenic lines without repeating *Agrobacterium* infiltration procedure in every production cycle (Buyel et al., 2017; Garabagi et al., 2012; Ma et al., 2015; Sack et al., 2015).

Despite this cost saving advantage, transient expression system remained preferable compared to stable expression system due to its higher expression level (Melnik et al., 2018). Low expression level in stable transgenic line is contributed mainly due to 'positional effect' and gene silencing mechanism (Dugdale et al., 2014; Rajeev Kumar et al., 2015). In this study, the concentration of anti-toxoplasma IgG transiently expressed in *N. benthamiana* (276.61 ± 9.52 µg/g fresh weight leaf) (Figure 4.30; Section 4.6.1) was comparable with the highest concentration of anti-toxoplasma IgG that was stably expressed in NT5.31 transgenic line (256.93 µg/g fresh weight leaf) (Figure 4.53; Section 4.8.6). However, it should be noted that the average concentration of anti-toxoplasma IgG stably expressed in the sampled T_1 generation was only 112.45 ± 10.01 µg/g fresh weight leaf (Section 4.8.6), ~60% lower than that of transient expression in *N. benthamiana*. Superiority of transient expression in *N. benthamiana* than stable expression in *N. tabacum* cv. SR1 has also been recently reported by Melnik et al. (2018).

Furthermore, among all of the expression systems that were evaluated in this study, the highest concentration of anti-toxoplasma IgG was recorded from transient expression system of *M. bracteata* (591.10 ± 52.5 µg/g fresh weight leaf) (Figure 4.27; Section 4.5.3). The antibody yield from transient expression in *M. bracteata* was twofold and fivefold higher than transient expression in *N. benthamiana* (276.61 ± 9.52 µg/g fresh weight leaf) (Figure 4.30; Section 4.6.1) and stable expression in *N. tabacum* cv. SR1 (112.45 ± 10.01 µg/g fresh weight leaf) (Figure 4.53; Section 4.8.6), respectively. Based on several published articles addressing the techno-economic principles of bio-manufacturing facilities, higher expression level of recombinant protein is paramount as it equates to significant increase in profitability (Buyel et al., 2017; Melnik et al., 2018; Nandi et al., 2016; Tusé et al., 2014; Walwyn et al., 2015).

CHAPTER 6: CONCLUSION

In conclusion, *M. bracteata* was able to express anti-toxoplasma IgG at twofold higher concentration compared to *N. benthamiana* as the model plant for transient expression system. In addition, *M. bracteata* transient expression system was superior to the stable expression system in *N. tabacum* cv. SR1 with an antibody yield that was fivefold higher and shorter production period. Thus, it could be recommended that a thorough techno-economic analysis for *M. bracteata* is indispensable as the next step to consolidate its potential as a production platform for recombinant protein production in an industrial setting.

Prior to evaluation of the different production platforms, reproducible protocols for *in vitro* propagation and transient expression of heterologous protein via *Agrobacterium*-mediated transformation in *M. bracteata* have also been successfully established. DNA content and genome size estimation reported provides base line data for future molecular cytogenetic study of *M. bracteata.* These protocols have a wide potential to facilitate application of –omics technologies in the future. Overall, this study has laid the foundation towards establishing *M. bracteata* as a viable platform with high potential for the production of recombinant pharmaceutical proteins.

Moving forward, several issues in particular regeneration of transgenic shoots to generate stable transgenic lines in *M. bracteata* remains as a major hurdle in exploring the full potential of this understudied crops. Recent successful strategies in recovering transgenic plantlets particularly from legume species have to be replicated with *M. bracteata*. Structural heterogeneity of heterologous protein in *M. bracteata* highlighted the needs to elucidate the glycosylation patterns or proteolytic degradation in *M. bracteata* in future studies with the final aim of humanizing the necessary glycosylation pathways in *M. bracteata*.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

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Abd-Aziz, N., Tan, B. C., Rejab, N. A., Othman, R. Y., & Khalid, N. (2020). A new plant expression system for producing pharmaceutical proteins. *Molecular Biotechnology, 62*, 240-251.

Aziz, N. A., Tan, B. C., Othman, R. Y., & Khalid, N. (2018). Efficient micropropagation protocol and genome size estimation of an important cover crop, *Mucuna bracteata* DC. ex Kurz. *Plant Cell, Tissue and Organ Culture, 132*(2), 267-278.

PAPERS PRESENTED

Aziz, N. A., Tan, B. C., Othman, R. Y., & Khalid, N. (2018). *Transient production of recombinant pharmaceutical protein in Mucuna bracteata.* Oral presentation at the 23rd Biological Sciences Graduate Congress, Dec 18th-20th 2018, Bangkok, Thailand.

Aziz, N. A., Tan, B. C., Othman, R. Y., & Khalid, N. (2017). *Transient production of recombinant pharmaceutical protein in Mucuna bracteata.* Poster presentation at the 2nd International Conference on Molecular Biology and Biotechnology, Nov 1st-2nd 2017, Kuala Lumpur, Malaysia.

Aziz, N. A., Tan, B. C., Othman, R. Y., & Khalid, N. (2015). *In vitro propagation of Mucuna bracteata, an important cover crop species in the tropics*. Poster presentation at the 22nd Annual Malaysian Society for Molecular Biology & Biotechnology (MSMBB), Sep 8th-9th 2015, Kuala Lumpur, Malaysia.