PRELIMINARY ASSESSMENT OF CONDITIONS INFLUENCING *Agrobacterium rhizogenes*-MEDIATED TRANSFORMATION OF *Eurycoma longifolia* JACK. SOMATIC EMBRYOS

BHAVANI BALAKRISHNAN

UNIVERSITI SAINS MALAYSIA

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by

BHAVANI BALAKRISHNAN

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
Adh	Alcohol dehydrogenase
ADP	Adenosine diphosphate
AM	Arbuscular mycorrhiza
ANOVA	Analysis of variance
aux	Auxin gene
BAP	6-Benzylaminopurine
bp	Base pairs
BW	Body weight
CaMV	Cauliflower Mosaic Virus
CAT	Chloramphenicol acetyltransferase
CDC	Centers for Disease Control and Prevention
cm	Centimetre
Co	Company
CSEM	Conventional scanning electron microscopy
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

DTT	Dithiothreitol
ED	Erectile dysfunction
EDTA	Ethylenediaminetetraacetic acid
et al.	*et alia*
EtBr	Ethidium bromide
EHT	Extra high tension
ESEM	Environmental scanning electron microscopy
FAA	Ethanol: glacial acetic acid: formaldehyde
FAS	Felda Agricultural Services
Fig	Figure
gusA	Beta glucuronidase gene
HR	Hypersensitive response
IAA	Indole-3-acetic acid
int	Intron
IBA	Indole-3-butyric acid
LOH	Late onset hypogonadism
LB	Luria Bertani
MS	Murashige and Skoog
NAA	Naphthalene acetic acid

No	Number
NOF	National Osteoporosis Foundation
nptII	Neomycin phosphotransferase II
OD	Optical density
OD_{600nm}	Optical density at 600nm
OPG	Osteoprotegerin
PCR	Polymerase chain reaction
PGRs	Plant growth regulators
PVP	Polyvinylpyrrolidone
Ri	Root inducing
RNA	Ribonucleic acid
rol	Root oncogenic loci
ROS	Reactive oxygen species
SEM	Scanning electron microscope
SD	Standard deviation
Sp	Species
Taq	*Thermos aquaticus*
TBA	Tertiary butyl alcohol

TBE	Trishydroxymethylaminomethane-borate-ethylenediaminetetraacetic acid
T-DNA	Transferred deoxyribonucleic acid
TEM	Transmission electron microscope
T_L-DNA	Left handed transferred deoxyribonucleic acid
TM	Trademark
TNT	2,4,6-Dinitrotoluene
T_R-DNA	Right handed transferred deoxyribonucleic acid
Tris HCL	Trishydroxymethylaminomethane-hydrochloride
TSS	Transfer stimulator sequences
uidA	Beta glucuronidase gene
UK	United Kingdom
USA	United States of America
USM	Universiti Sains Malaysia
UV	Ultraviolet
VP SEM	Variable pressure scanning electron microscope
vir	Virulence genes
WHO	World Health Organization
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid

LIST OF SYMBOLS

°C	Degree Celcius
/	Division/or
=	Equal to
g	Gram
g/L	Gram per litre
L	Litre
M	Molar
µ	Micro
µg	Microgram
µg/L	Microgram per litre
µl	Microlitre
µm	Micrometre
µM	Micromolar
$\mu mol.m^{-2}.s^{-1}$	Micromole per metre square per second
mg	Milligram
mg/kg	Milligram per kilogram
mg/L	Milligram per litre

mL	Millilitre
mM	Milimolar
mm	Millimetre
-	Minus
nm	Nanometre
ng	Nanogram
Pa	Pascal
%	Percentage/Percent
+	Plus
±	Plus or minus
psi	Pound per square inch
rpm	Revolution per minute
cm^2	Square centimetre
×	Times
U	Unit
V	Volt
v/v	Volume over volume
W	Watt
w/v	Weight over volume

PENILAIAN AWAL KONDISI YANG MEMPENGARUHI TRANSFORMASI EMBRIO SOMATIK *Eurycoma longifolia* Jack. OLEH *Agrobacterium rhizogenes*

ABSTRAK

Propagasi *in vitro* yang berkesan untuk kultur kalus, pucuk, anak tumbuhan dan embrio somatik bagi *Eurycoma longifolia* telah diperolehi dengan menggunakan teknik mikropropagasi. Kultur pucuk, embrio somatik dan anak tumbuhan *in vitro* telah digunakan dalam analisis histologi dan SEM, asai kemotaksis dan transformasi dengan empat strain terlucut *Agrobacterium rhizogenes* yang berlainan. Analisis histologi serta pemerhatian SEM menunjukkan bahawa embrio somatik *Eurycoma longifolia* mempunyai ciri-ciri yang sama dengan embrio zigotik. Respon kemotaksis yang positif oleh *Agrobacterium rhizogenes* terhadap eksplan embrio somatik telah dikesan dengan menggunakan asai piring kerumun. Kultur pucuk dan anak tumbuhan *in vitro* telah digunakan untuk memperolehi eksplan daun, batang dan rakis bagi transformasi. Struktur berbentuk akar rerambut didapati muncul dari eksplan batang *in vitro* yang dikultur bersama dengan *Agrobacterium rhizogenes* strain AR14. Walau bagaimanapun, bilangan eksplan yang menunjukkan struktur berbentuk akar rerambut adalah terlalu sedikit untuk dikenal pasti melalui asai GUS atau pengesanan transgen berdasarkan PCR. Pengoptimuman transformasi dengan eksplan embrio somatik telah dikendalikan dengan menganalisis kesan empat faktor yang berlainan terhadap frekuensi positif GUS. Adalah dipastikan bahawa AR12 merupakan strain yang paling virulen dan sesuai untuk transformasi embrio somatik *E. longifolia*. Formulasi media yang diperolehi adalah seperti berikut: Media MS berkuatan penuh yang ditambah dengan 1.0 mg/L IBA, 1% (w/v) PVP, 3% (w/v) sukrosa dan

dipejalkan dengan 2.8 g/L Gelrite^{TM}. Formulasi media ini menunjukkan frekuensi GUS positif sebanyak 94.2%. Integrasi stabil gen *rolA*, *rolB*, *rolC* dan *virD* dari *Agrobacterium rhizogenes* dalam *Eurycoma longifolia* telah disahkan menggunakan analisis molekular berasaskan reaksi rantai polimerasi (PCR) dengan amplifikasi produk PCR pada 248 bp, 780 bp, 490 bp dan 450 bp masing-masing. Akan tetapi, induksi akar rerambut tidak diperolehi dalam embrio somatik yang telah ditransformasi.

PRELIMINARY ASSESSMENT OF CONDITIONS INFLUENCING *Agrobacterium rhizogenes*-MEDIATED TRANSFORMATION OF *Eurycoma longifolia* Jack. SOMATIC EMBRYOS

ABSTRACT

Effective propagation of *in vitro* callus, shoot, plantlet and somatic embryo cultures of *Eurycoma longifolia* was achieved using micropropagation technique. *In vitro* shoots, somatic embryos and plantlet cultures were used for conducting histological and SEM analysis, chemotaxis assays and transformation with four different *Agrobacterium rhizogenes* disarmed strains. Histological analysis and SEM observations revealed that somatic embryos of *Eurycoma longifolia* share similar traits with zygotic embryos. Positive chemotactic responses of *A. rhizogenes* towards somatic embryo explants were detected using swarm plate assay. *In vitro* shoot and plantlet cultures were used to obtain leaves, stems and rachis explants for transformation. Hairy root-like structures were seen emerging from *in vitro* stem explants co-cultivated with *Agrobacterium rhizogenes* strain AR14. However, the number of explants expressing these hairy root-like structures was too low to be confirmed using GUS assay or PCR based detection of transgenes. Transformation optimisations with somatic embryo explants were conducted by analysing the effect of four different factors on the GUS positive frequency. It was ascertained that AR12 is the most virulent and suitable *Agrobacterium rhizogenes* strain for transformation of *E. longifolia* somatic embryos. The medium formulation achieved is as follows: Full strength MS medium added with 1.0 mg/L IBA, 1% (w/v) PVP, 3% (w/v) sucrose and solidified with 2.8 g/L Gelrite^{TM}. This medium formulation showed a GUS positive frequency of 94.2%. The stable integration of *rolA*, *rolB*, *rolC* and

virD genes from *Agrobacterium rhizogenes* in *Eurycoma longifolia* were confirmed using polymerase chain reaction (PCR) based molecular analysis with the amplification of PCR products at 248 bp, 780 bp, 490 bp and 450 bp, respectively. However, the induction of hairy roots was not achieved in transformed somatic embryos.

CHAPTER 1

INTRODUCTION

The first pharmacologically active compound was sequestered from *Papaver somniferum*, the poppy plant, by a young pharmacist called Friedrich Sertümer a little over 200 years ago. The discovery of morphine heralded the age of modern pharmaceuticals wherein plant-based drugs could be refined, analysed and administered in accurate amounts that did not fluctuate with the source of the substance. By the mid of the 20th century, pharmaceutical explorations had grown to encompass the prodigious screening of microorganisms for antimicrobial compounds due to the accidental discovery of penicillin from the ascomycetous fungi, *Penicillium* (Jesse and Vederas, 2009). "The remedies are in our own backyard", said the eminent cover of the *TIME* magazine upon the astonishing discovery of streptomycin from a lump of mold from a chicken farmer's backyard (Mukherjee, 2011).

Four decades later, natural products and analogs inspired by them made up 80% of the commercial drugs in the market. By then, the advancement of high-throughput screening techniques had granted the ability to accelerate the search for pure synthetic compounds, allowing the encroachment of synthetic medicinal chemistry into the pharmaceutical industry. Detection of natural products began to dwindle which prompted the question: is the age of discovery of new drugs from natural sources ending? (Jesse and Vederas, 2009).

Studying the socio-cultural anthropology in these parts of the world reveals the answer to this question to be an emphatic "no". The use of folk medicine derived from natural sources is entrenched in Asian culture, passed down by the word of

mouth and practiced widely on a daily basis. Widely recognised locally by the name of Tongkat Ali, *Eurycoma longifolia* is arguably one of the most famous plants in Southeast Asia. Literally translated as 'Ali's cane', the reputation of this Simaroubaceous shrub-tree precedes itself. Its various plant parts have been shown to possess numerous compounds with medicinal properties with a special emphasis on its root, believed to cure sexual insufficiency in men suffering from erectile dysfunction (Kuo *et al.*, 2004).

Concerns on declining populations, the loss of genetic heterogeneity, habitat deterioration and local extinctions have been triggered by the haphazard collection methods of the taproot from the wild (Canter *et al.*, 2005; Sobri *et al.*, 2005b). Low initiation rates and long germination periods by the immature zygotic embryos of *E. longifolia* compound matters further. This pressing need of the herbal and pharmaceutical industry is driving the demand for the prompt mass multiplication of the roots on a commercial scope (Sobri *et al.*, 2005b).

The development of plant tissue culture techniques has allowed scientists to conserve and protect many endangered species from the advent of rapid modernisation and habitat destruction. However, the recalcitrant nature of this woody plant has been a stumbling block in the path to producing sustainable root cultures in bioreactors for research as well as commercial purposes. Adventitious root cultures have been developed using tissue culture manipulations aided by phytohormones (Sobri *et al.*, 2012). However, these methods may not be commercially viable due to the cost incurred during up-scaling while the amount of rooting achieved is well below 50%. Alternative methods for producing root cultures are vital for the long term sustainability of such endeavours.

The proposed approach was to utilise the naturally-occurring soil bacterium *Agrobacterium rhizogene*s, known as nature's genetic engineer and the formative agent of hairy root condition in dicotyledonous plants (Veena and Taylor, 2007). The relocation of T-DNA into the plant genome is directed by the root-inducing (Ri) plasmid following which the assimilation and execution of the various translocated genes occurs. Characterised by swiftly-growing, non-geotropic, strikingly branching growth on basal medium (Christey, 2001) this technique of root induction would increase root biomass for a taproot plant such as *E. longifolia*. The fast growth rates of hairy root cultures on medium free of hormones achieves the dual target of satisfying consumer demands while significantly reducing production costs.

The primary aim of this research is to a conduct a preliminary assessment of the various factors influencing the induction of hairy root cultures from *Eurycoma longifolia*. The new knowledge gained would help scientists to understand a culturally valuable medicinal plant while developing tools to help the conservation efforts and bringing it one step closer to mass commercialization.

1.1 Research objectives

The objectives of this research project were:

- To micropropagate *Eurycoma longifolia* in a controlled tissue culture environment and study the features that makes them suitable for *Agrobacterium rhizogenes*-mediated transformation,
- To study the interaction between *Agrobacterium rhizogenes* and *Eurycoma longifolia* leading to a successful transformation,
- To optimise genetic transformation protocol for *Eurycoma longifolia* mediated by *Agrobacterium rhizogenes*,
- To determine the successful and stable incorporation of transgene into the plant genome using PCR analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 *Eurycoma longifolia*: Locality and tree morphology

Eurycoma longifolia Jack., occasionally cited as the 'Malaysian ginseng' (Jagananth and Ng, 2000) is an affiliate of the Simaroubaceae family indigenous to Southeast Asia. Its common names include 'Tongkat Ali', 'Pasakbumi', 'Ian-don' and 'Cay ba binh' in Malaysia, Indonesia, Thailand and Vietnam, respectively. To the casual observer, the plant appears as a lanky tree that grows 500 metres above the sea level in lowland forests as an understory (Ang *et al.*, 2002; Choo and Chan, 2002; Kuo *et al.*, 2004).

Few branches are often observed on the evergreen, slow growing tree that could grow up to 15 to 18 metres with dark green pinnate leaves arranged spirally around its stem (Shuid *et al.*, 2011). The dioecious plants form their densely-arranged flowers on large, branched, hairy panicles that arise from leaf axils and ranges 60 to 70 cm in length. Generally, two to three years of cultivation is required for the trees to develop the reddish brown drupe fruits. The seeds often germinate after about a month. Conventional methods of harvesting usually include pulling out the whole plant to obtain the tap root after approximately four years of cultivation (Jagananth and Ng, 2000). Sandy, well drained soil supplemented with sufficient water and partial shade conditions promotes the abundant growth of the plant (Bhat and Karim, 2010).

2.2 Ethnobotanical practices in native communities

As is customary for plants with a prolonged history of medicinal usage in Asia (Sobri *et al.*, 2012), it has often been recommended either as a standalone ingredient or as a concoction with other herbs in folk medicine. Various plant parts are used for the treatment of sexual inadequacy, malarial infections, aches, pyrexia, diarrhea and glandular swelling (Kuo *et al.*, 2004). It is believed to relieve fatigue and improve energy levels, and is consumed as a health tonic (Sobri *et al.*, 2012). Lesser known medicinal uses of the plant comprise the treatment of lumbago, cachexia, oedema, rheumatism and arthritis (Jagananth and Ng, 2000).

Postnatal women are often dispensed with the root extract as a herbal supplement to enhance blood flow (Ismail *et al.*, 1999). Traditional drug preparation methods are used to prepare the roots for consumption, wherein they are cut into small portions, boiled and drunk as a tea. Oftentimes, intense boiling in fresh water and the addition of honey or sugar syrup are suggested to lessen the bitterness of the root. Consumption of alcohol which has been incubated with the root powder has also been observed although some healers believe that the bitterness boosts the potency of the brew (Bhat and Karim, 2010).

2.3 Elucidation of compounds and pharmacological properties

These ethnobotanic uses of *Eurycoma longifolia* have triggered the interests of scientists to determine the validity of these claims empirically. The roots have been found to be a valuable source of quassinoids (Ang *et al.*, 2000; Teh *et al.*,

2010), β-carboline (Kuo *et al.*, 2003) and canthin-6-one alkaloids (Kardono *et al.*, 1991). Some of these constituents were shown to inhibit plant growth (Jiwajinda *et al.*, 2001) and possess anti-tumour and anti-parasitic (Jiwajinda *et al.*, 2002), cytotoxic (Kuo *et al.*, 2003), anti-plasmodial (Chan *et al.*, 2004) and anti-hyperglycemic activities (Husen *et al.*, 2004).

2.3.1 Malaysian Ginseng

Approximately 22% of Malaysian men between 40-70 years have been reported to experience erectile dysfunction, rendering it a highly prevalent sexual complication (Nicolosi *et al.*, 2003). Due to the nature of this health problem and its associated social stigma, many sufferers fail to seek adequate and timely medical treatments. However, some appear to be amenable towards herbal remedies (Low and Tan, 2007). Traditionally, *Eurycoma longifolia* is most famously used to treat sexual insufficiency (Kuo *et al.*, 2004) or increase sexual prowess (Ang and Cheang, 1999a). Multiple scientific studies have been conducted to determine the legitimacy of this application.

Sexual impetus and copulatory habits in test rat subjects were altered by the *E. longifolia* extract. Extended period of coitus and shorter obstinacy span were observed in male rats treated with *E. longifolia* fractions (Ang and Sim, 1997). In an elegant experiment designed to study sexual inclination in male rats, both experimental and control groups were placed in electrical copulation cage. The experimental group was fed with *E. longifolia* fractions and observed for their

willingness to overcome aversive stimulus to mate with potential female partners in the coupling cage. Treated subjects showed a high level of successful transversions, mountings, admittance and copulation that were not observed among their control counterparts (Ang and Sim, 1998). A significant increase in orientation activities, genital grooming (Ang and Lee, 2002) and a decrease in hesitation time were observed in treated middle aged male rats (Ang *et al.*, 2003).

Physical alterations were also observed as the extract feeding promoted the improvement of sexual appendage by increasing the weight of leavator ani muscle (Ang and Cheang, 2001) and enhancing penile response (Ang *et al.*, 2001). Sperm count, motility and viability were increased in male rats fed with water extracts of *E. longifolia* (Noor *et al.*, 2004) while treating sexually lethargic male rats using root powder increased testosterone serum levels (Zanoli *et al.*, 2009). An increase in spermatogenesis and sperm count were reported for male rats treated with estradiol, suggesting that compounds found in extracts of *E. longifolia* exert anti-estrogenic activity (Wahab *et al.*, 2010) while female rats with irregular oestrous cycles experienced less follicular morphological damage (Abdulghani *et al.*, 2012). Eurycomanone displayed comparable potency to tamoxifen and was proposed as a treatment for uterotrophy of immature rats (Teh *et al.*, 2011).

Although many studies have been conducted on the effects of *E. longifolia* on rat models, clinical trials involving human subjects are severely lacking. Overall enhancement of sperm motility, morphology, concentration and the amount of semen were noted in male patients suffering from infertility when provided with water extract of *E. longifolia* root (Tambi and Imran, 2010). These results were concurred by another study in which 30-55 year old men treated with water extract reported improvements in overall erectile function, sexual libido, sperm motility and semen

volume (Ismail *et al.*, 2012). Clinical evaluation of water extract of *E. longifolia* root revealed a reduction in symptoms of late-onset hypogonadism (LOH) and normal testosterone levels in treated patients (Tambi *et al.*, 2012).

2.3.2 Antiparasitic activities

Malaria is transmitted via the bites of mosquitoes infected with a parasite called *Plasmodium* ("Malaria," 2013). Approximately 219 million cases of malaria were reported worldwide in 2010, resulting in 660,000 deaths, mostly on the African continent ("Malaria," 2012). Parasitic resistance to malarial medicine is a very real problem in many parts of the world ("Malaria," 2013). Therefore, the search for new and potent compounds to combat the spread of malaria must be commenced immediately.

Methanol-soluble root extracts of *Eurycoma longifolia* containing 7-methoxy-β-carboline-1-propionic acid and eurycomanone showed significant antimalarial activity when tested against cultured *Plasmodium falciparum* strains (Kardono *et al.*, 1991). Eurycomanol, eurycomanol-2-*O*-β-D-glycopyranoside and 13β, 18-dihydroeurycomanol showed potency against chloroquine-resistant *P. falciparum* strains from Malaysia (Ang *et al.*, 1995). Potent plasmocidal activity was also recorded for two quassinoid compounds, 11-Dehydroklianeanone and 15β-O-acetyl-14-hydroxyklianeanone derived from *E. longifolia* leaves (Jiwajinda *et al.*, 2002). The protein synthesis in *Plasmodium falciparum* is inhibited by quassinoids, which results in their effectiveness at halting the growth of the parasite during treatments (Tran *et al.*, 2003). As a remedy against malarial fever, the aqueous extract of

Eurycoma longifolia was found to be as potent as the dichloromethane extract (IC50 ≤ 4 µg/mL) which affirms the claims by traditional practitioners on antimalarial usage of the plant (Hout *et al.*, 2006).

Schistosomiasis is one of the most widespread parasitic infections in the world. Currently, Praziquantel is used to treat millions of people infected with schistosomiasis annually and drug-resistant parasites are likely to evolve (Esmat and Raziky, 2009). Longilactone, a quassinoid from *Eurycoma longifolia* leaves were found to possess antischistomal effect with an IC_{50} of 200 µg/mL (Jiwajinda *et al.*, 2002).

Approximately 30% of the global human population is plagued by *Toxoplasma gondii,* a protozoan parasite found in tropical and subtropical climates. Severe neurological deficits in immunosuppressed patients and lymphadenopathy in healthy adults is often observed as a result of the infection. *Eurycoma longifolia* root fractions were found to possess potential anti-*Toxoplasma gondii* activity (Kavitha *et al.*, 2012a). Scanning electron microscopy (SEM) observations showed changes in cell wall leading to thoroughly collapsed cells compared to the untreated cells. A reduction in cytoplasmic volume, structural disorganization and devastated organelles were observed using transmission electron microscopy (TEM) after 12 hours of treatment, indicating a speedy antiparasitic activity (Kavitha *et al.*, 2012b).

2.3.3 Cytotoxic properties

Cancer is an insidious disease in which cells have acquired an autonomous will to divide leading to uncontrollable, pathological cell division. Cancer is predicted to eclipse heart disease to become the most common cause of death with approximately 15% of mortality rates being ascribed to cancer (Mukherjee, 2011). These grave forecasts have made it absolutely imperative to seek and develop new antiproliferative drugs with little to no toxicity to healthy cells.

Biochemical analyses conducted on the roots and leaves of *Eurycoma longifolia* have shown that it possesses potent cytotoxic activities. Eurycomalactone, eurycomanone, 14,15β-dihydroxyklaineanone, 6-dehydroxy-longilactone, canthin-6-one 9-*O*-β-glucopyranoside, 9-methoxycanthin-6-one and pasakbumin B and C demonstrated compelling reaction against both the MCF-7 and A-549 cell lines, corresponding to human breast and lung cancers respectively (Kuo *et al.*, 2004). The inhibition of Epstein-Barr virus activation and the enhancement of anti-cancer activity were reported for the quassinoid compound 14,15β-dihydroxyklaineanone extracted from the leaves (Jiwajinda *et al.*, 2002).

Cytotoxic activities by the methanolic and aqueous extracts of E .longifolia were shown against mouse FM3A mammary carcinoma cells (Tran *et al.*, 2003). However, conflicting reports on the use of aqueous extracts do exist. When methylene blue assay was used to compare water, chloroform, *n*-butanol and methanol extracts derived from *Eurycoma longifolia* Jack. root for their cytotoxic effect, all extracts except water showed convincing outcome on the cell lines (Nurhanan *et al.*, 2005).

Eurycomanone was shown to elicit apoptotic response in human cervical carcinoma (HeLa) cells by up-regulating Bax pro-apoptotic and tumor suppressor p53 proteins while Bcl-2 anti-apoptotic protein was down-regulated. The treatment resulted in the condensation of chromatin structure, DNA fragmentation and formation of apoptotic bodies. It is also relatively non-toxic to normal (MDBK and Vero) cells (Mahfudh and Azimahtol, 2008). In another study, hepato carcinoma cells, HepG2 were found to be susceptible to eurycomanone by inducing apoptosis in cancerous cells as demonstrated by the increase in cytochrome c levels in the cytosol (Zakaria *et al.*, 2009). Strong cytotoxic activity by longilactone was exerted on the human breast cancer cell line, MCF-7 by inducing apoptosis and activating the caspase-7,-8 and poly (ADP-ribose) polymerase. However, the basal levels of Bcl-2 and Bax proteins were not influenced by longilactone (Muhamad *et al.*, 2011). These studies indicate that eurycomanone and longilactone are promising cytotoxic chemotherapeutic agents.

2.3.4 Preventing bone loss and osteoporosis

Microarchitectural deterioration and low bone mass resulting in elevated risk of fracture and the development of bone fragility characterises osteoporosis (*Clinician's guide to prevention and treatment of osteoporosis*, 2010). The aggravation of sleep apnoea, erythrocytosis, cardiovascular disease, liver damage and heightened risk for prostate cancer have been correlated with testosterone replacement therapy used to treat patients suffering from osteoporosis (Rhoden and Morgentaler, 2004; Bhasin *et al.*, 2006). Therefore, an alternative treatment that possesses lesser side effects compared to the conventional therapy while

concurrently providing protection for the bone is necessary and essential (Ramli *et al.*, 2012).

Standardised aqueous extract of *Eurycoma longifolia*, containing the bioactive compounds eurycomanone, glycosaponin and eurypeptide were tested on orchidectomised (androgen-deficient osteoporosis model) rats. Micro-CT analysis indicates that high dose of *Eurycoma longifolia* may protect bone microarchitecture. However, the study also found that lower doses could potentially exacerbate osteoporotic deterioration (Ramli *et al.*, 2012). In a similar study on orchidectomised rats, supplementation with *E. longifolia* extract diminished the bone resorption marker, invigorated testosterone levels and up-regulated osteoprotegerin (OPG) gene expression. The insulating effects of the extract against bone resorption were attributed to these factors (Shuid *et al.*, 2012).

2.3.5 Other properties

Hyperglycemia resulting from the lack of or reduced sensitivity to glucose characterises diabetes mellitus (Greenberg and Sacks, 2002). The blood glucose reducing effect of water extract of *Eurycoma longifolia* were used to treat normoglycemic and Streptozotocin-induced hyperglycaemic rats which yielded positive antihyperglycemic results (Husen *et al.*, 2004).

Oxidative damage caused by free radicals could be prevented by antioxidants and have been suggested for use in anti-inflammatory and cardiovascular diseases. When *E. longifolia* extract were assayed for chelating effect on several free radicals, moderate antioxidant activity have been found and reported (Suhartonoa *et al.*, 2012).

Methanolic extracts of *E. longifolia* roots were fed orally to inbred adult albino mice and were evaluated for the emotional state of the test organisms. Reduced fighting episodes similar to the antianxiety effects of the commercial drug diazepam were found in treated subjects, advocating the use of the plant for anxiety treatment (Ang and Cheang, 1999b). More recently, the effect of *E. longifolia* water extract on cognitive disposition and stress hormones in moderately stressed human subjects were tested. It revealed significant improvements for tension, anger and confusion states with elevated testosterone status and lesser cortisol exposure (Talbott *et al.*, 2013).

2.4 The consequences of fame on *Eurycoma longifolia*

The high commercial value of *E. longifolia* is primarily due to the aphrodisiac property which has led to a high demand in the market. Food supplements, tablets and health drinks make up the most sought-after products of *E. longifolia* (Sobri *et al.*, 2012). The population of *E. longifolia* is dwindling in the wild due to haphazard commercial collections and the steady increase in demand from consumers. Seed germination, cutting and other conventional propagation methods are insufficient to satisfy the requirement in the market today. The maturation process of the plant takes about five years with low fruit harvest (Chang, 2000), resulting in slow and low reproduction rates. The development of root cultures using plant tissue culture techniques could be an alternative method to obtain raw materials of *E. longifolia* for the industry (Sobri *et al.*, 2012).

2.5 Current *in vitro* propagation methods of *Eurycoma longifolia*

In the last decade, various *in vitro* cultures have been established for *Eurycoma longifolia*. Friable callus obtained from leaf explants of *E. longifolia* and cultured in MS (Murashige and Skoog, 1962) liquid medium containing 1-naphthaleneacetic acid (NAA) and 2, 4-dichlorophenoxyacetic acid (2,4-D) produced the best suspension cells (Siregar, 2004). Cotyledon explants were induced into embryogenic callus in basal MS medium containing kinetin, 2,4-D and activated charcoal. The mature somatic embryos were then induced into plantlets by transferring into MS medium complemented with kinetin (Sobri *et al.*, 2005a). MS medium with kinetin were also used to culture *in vivo* shoot tip explants and rooting of shoot cultures were achieved with the addition of indole-3-butyric acid (IBA) (Sobri *et al.*, 2005b). Leaf explants were induced to generate adventitious roots by culturing in MS medium containing NAA and high concentration of sucrose (Sobri *et al.*, 2012). However, another study reported that the optimal number of shoots and roots from *in vitro* germinated seeds was achieved in MS medium with 6-benzylaminopurine (BAP) and IBA, respectively (Hassan *et al.*, 2012). These conflicting reports may be attributed to genotypic variability and the type of explants used for the study. Investigations had also been carried out to determine the optimal medium formulations for achieving the highest cell biomass and alkaloid production in cell suspension cultures (Siregar *et al.*, 2003). Factors influencing 9-methoxycanthin-6-one aggregation in callus cultures were studied and MS medium with sucrose, dicamba and phenylalanine were reported to give the highest yield of the metabolite (Rosli *et al.*, 2009).

2.6 *Agrobacterium rhizogenes*: Nature's genetic engineer

The Gram negative soil bacterium, *Agrobacterium rhizogenes*, also known as *Rhizobium rhizogenes* and the root-inducing (Ri) plasmid found within the cell is culpable for the hairy root symptoms displayed by infected dicotyledonous plants (Veena and Taylor, 2007). The plasmid directs T-DNA relocation, assimilation and expression following the infection of injured sites by *Agrobacterium rhizogenes* (Christey, 2001).

The T-DNA fragment is flanked by 25-bp direct repeats, which act as a *cis* element signal for the transfer apparatus. The process of T-DNA transfer is mediated by the cooperative action of proteins encoded by genes determined in the Ri plasmid virulence region (*vir* genes) and in the bacterial chromosome (De la Riva *et al.*, 1998). The 30 kb virulence (*vir*) region is a regulon organized in six operons that are essential for the T-DNA transfer (*virA, virB, virD,* and *virG*) and for increasing the transfer efficiency (*virC* and *virE*) (Hooykaas and Shilperoort, 1992; Zupan and Zambryski, 1995; Jeon *et al.*, 1998). The transformation process begins with recognition of plant signals by the bacterial VirA/VirG sensory system, followed by activation of the *vir* loci and attachment of the bacterium to the host cell. The T-strand is excised from the T-DNA region by VirD2/VirD1 and exported, *in cis* with a covalently attached VirD2 molecule and *in trans* with several other Vir proteins, into the plant cell cytoplasm via a VirB/D4 type IV secretion system. Inside the host cell, the VirD2–T-strand conjugate is packaged by numerous molecules of VirE2 to form a mature T-complex (Citovsky *et al.*, 2007) that would facilitate the passage of the T-strand into the nuclear pore (Dumas *et al.*, 2001). VirD2 may also

play a role in the integration of T-DNA into the plant genome. Various mutations in VirD2 can affect either the efficiency (Mysore *et al.*, 1998) or the "precision" (Tinland *et al.*, 1995) of T-DNA integration.

Agropine, mannopine, cucumopine, and mikimopine-type strains are the most frequently isolated *A. rhizogenes* strains. The polarity of infection on plant tissues distinguishes these strains from each other. The variation between the polar and non polar types may be dictated by the presence of a second T-DNA segment in agropine type strains that codes for auxin synthesis and transport mechanism (Meyer *et al.*, 2000) in transformed plants, which may account for its non polar nature. Agropine, the only non polar type strain, is capable of eliciting the induction of hairy roots irrespective of disc orientation (Veena and Taylor, 2007).

There are several differences between *Agrobacterium rhizogenes* and its cousin, *Agrobacterium tumefaciens*. The T-DNA transfer stimulator sequences (TSS) encoded in *A. rhizogenes* are thought to increase the translocation competency of the Ri plasmid (Moriguchi *et al.*, 2001). The lack of *vir*E1 and *vir*E2 genes in the *A. rhizogenes* genome have been replaced by the GALLS gene which substitutes for *vir*E2 function in *A. tumefaciens* (Hodges *et al.*, 2004; Veena and Taylor, 2007). T_L-DNA encoded *rol* (root oncogenic loci) genes comprising of *rolA*, *B*, *C* and *D* are required for hairy root induction (Estramareix *et al.*, 1986; Slightom *et al.*, 1986; Veena and Taylor, 2007). The mechanism of action of these genes is by modulating the synthesis and sensitivity of the plant towards various hormones leading to a modification in plant growth (Jasik *et al.*, 1997; Veena and Taylor, 2007).

2.7 Hairy root phenotype

A diverse variety of plants could be used to induce hairy roots and these could often be generated into whole organisms. Characterised by speedy, non-geotropic, strikingly branching growth on basal medium (Christey, 2001), this technique of root induction would consequently increase root biomass for a taproot plant such as *E. longifolia*. The prolific growth of hairy roots is comparable to that of undifferentiated callus while retaining a functionally differentiated root organ (Veena and Taylor, 2007). The translocation and expression of *rol* genes have been shown to elicit modified phenotype in plants established from hairy roots which include anatomical shift such as diminished apical dominance, shorter internodes and wrinkled leaves (Christey, 2001; Christey and Braun, 2004).

2.8 *Agrobacterium rhizogenes*-mediated transformation of *Eurycoma longifolia*

Transferring genes into plant DNA by utilising bacterial vectors have been shown to enhance productivity and a competent transgene delivery system for *Agrobacterium rhizogenes* are already established for various valuable medicinal plants (Canter *et al.*, 2005). The 'hairy root phenotype' is often defined as an inordinately bountiful and non-geotropic root system (Veena and Taylor, 2007). The fast growth rates of hairy root cultures on basal medium achieve the dual target of satisfying consumer demands while significantly reducing production costs. The production of herbal medicines have several ingrained complications such as

variability of the source plant, irregular output of compounds and the presence of harmful elements that may be clarified with the development of uniform, high-quality clones with optimised yield (Canter *et al.*, 2005). Studies conducted on herbal preparations of *Polyalthia bullata* reveal that 26% of these products possessed high concentrations of toxic heavy metal mercury that did not comply with the threshold limit set by the Drug Control Authority (DCA), Malaysia (Ang and Lee, 2006). Hairy root induction using wild strains of *A. rhizogenes* had been reported for *in vitro* germinated seeds of *E. longifolia*. The roots were initiated by infecting the hypocotyl region of the three (3) week old germinated seedlings and they were thin, non-geotropic, hairy and brittle in appearance (Danial *et al.*, 2012). However, no reports have been published on the induction of hairy roots using disarmed strains of *A. rhizogenes* which are generally regarded as more virulent compared to wild strains and possess reporter genes for easy identification in transformed plants.

2.9 Potential applications for hairy root cultures of *Eurycoma longifolia*

Hairy roots and the plants regenerated from them have many uses in various fields of research. Some of the potential uses of the root cultures are as bioreactors for the production of valuable plant metabolites, to conduct fundamental research on metabolic pathways, plant-biotic communication mechanisms and to preserve valuable germplasms using cryopreservation techniques (Christey and Braun, 2004).

2.9.1 Genetic and metabolic engineering

Once an efficient and stable transformation protocol has been established, these tools could be utilised to engineer foreign genes into the plant genome or to alter their metabolic processes. By sidestepping the conventional methods of selective breeding, resistance to pathogens could be instilled in the plant within one generation. Using the composite plant establishment technique, cyst nematode resistance was engineered into the roots of soybean plants (Liu *et al.*, 2012).

By introducing genes that catalyse certain glycosylation, hydroxylation and methylation reactions, some metabolites could be selectively altered or secreted (Giri and Narasu, 2000), which could help engineer strategies to bypass obstacles linked to feedback regulation and availability of precursor compounds (Memelink, 2005). The *Cathranthus roseus*-derived tryptophan decarboxylase gene produced elevated amounts of nicotine and serotonin in engineered roots of *Peganum harmala* (Berlin *et al.*, 1993; Giri and Narasu, 2000).

2.9.2 Molecular farming: Phytochemicals, proteins and bioreactors

The growth and production of secondary metabolites from hairy root cultures have been shown to remain stable for a number of years when properly maintained and sub-cultured at regular intervals (Hamill and Lidgett, 1997). *Datura stramonium* hairy root lines synthesising tropane alkaloids remained at the same level (~1% dry

weight) for 5 years, representing about 75 subcultures (Baíza *et al.*, 1998). Hightened yields of several natural products were found in hairy root cultures compared to intact plants. The insertion of the genetic construct by *A. rhizogenes* could occasionally lead to the production of compounds not found in untransformed roots (Shanks and Morgan, 1999). For instance, glucose conjugates of flavonoids were accumulated in the untransformed roots of *Scutellaria baicalensis* while the transformed roots formed glucoside conjugates (Hu and Du, 2006). Although the synthesis of secondary metabolites in hairy roots is directed by genetic mechanisms, it is also guided by biotic and abiotic elements such as infection, stress, variations in pH, fluctuating temperature and light conditions (Canter *et al.*, 2005). Any modifications to these physical and chemical factors may trigger the formation of useful variants of pre-existing metabolites.

One area of hairy root research which garners an intense amount of commercial interest is the manufacturing of industrial and therapeutic proteins by plants using bioprocessing technology (Shanks and Morgan, 1999). A novel ribosome-inactivating protein secreted into the media was reported in the *A. rhizogenes* derived cell suspension culture of *Trichosanthes kirilowii*, making it favourable for product recovery (Shih *et al.*, 1998).

The scaling up and downstream processing of established root cultures is crucial for applications in industry (Guillon *et al.*, 2006). Oxygen limitation is often the preeminent constraint on the development of culture systems (Kanokwaree and Doran, 1997). Callus formation may result from injury caused by mechanical shaking and the branching causes the roots to exhibit a resistance to flow, further restricting successful distribution of nutrients. Various types of bioreactors have been proposed to overcome these problems such as submerged and mist bioreactors (Giri and

Narasu, 2000) and some have proven successful. The production of saponin from *Panax ginseng* hairy roots were achieved with airlift reactors (Yoshikawa and Furuya, 1987) while mist reactors were used to grow tobacco hairy roots containing mouse interleukin-12 (Liu *et al.*, 2009).

2.9.3 Functional gene analysis

Functional gene analysis could be conducted in transformed plants to gain in depth knowledge on their mechanisms of action. Roots expressing foreign genes could be used in loss of function studies by conducting transient RNA silencing. By engineering hairpin RNA sequences complimentary to the GUS coding region, the GUS activity in *Lotus japonicus* hairy roots were curtailed (Kumagai and Kouchi, 2003). GUS promoter function was studied by varying conditions such as wounding, temperature and abscisic acid treatments by fusing it with the alcohol dehydrogenase (*Adh*) promoter in hairy roots of soybean (Preiszner *et al.*, 2001).

2.9.4 Phytoremediation

The absorption and accumulation of heavy metals and the enzymatic change of toxic organic molecules to harmless forms by plants is called phytoremediation (Guillon *et al.*, 2006). Hairy roots have been proposed as an ideal choice to cleanse polluted soil or water (Shanks and Morgan, 1999). *Solanum nigrum* hairy roots have

been observed to remove polychlorinated biphenyls from the culture medium (Kas *et al.*, 1997). Through a series of reactions, *Catharanthus roseus* hairy roots have been able to reduce and incorporate the nitroaromatic explosive 2,4,6-dinitrotoluene (TNT) to an unextractable cell wall-bound residue (Bhadra *et al.*, 1999). In the future, genetically altered hairy root cultures could provide a viable alternative to current environmental clean-up technologies.

CHAPTER 3.0

MATERIALS AND METHODS

3.1 Micropropagation of *Eurycoma longifolia*

3.1.1 Callus induction from *in vivo* and *in vitro* leaf explants

Mature *in vivo* leaf explants were collected from *Eurycoma longifolia* plant grown at the School of Biological Sciences, Universiti Sains Malaysia (USM), Pulau Pinang. Using a modified surface sterilisation protocol (Rosli *et al.*, 2009), the explants were washed under running tap water and soaked in three (3) to four (4) drops of Tween-20 (Sigma) for 30 minutes. Subsequent steps were carried out in laminar flow chamber under aseptic condition. Three (3) drops of Tween-20 were added as surfactant into 30% (v/v) commercial Clorox solution prepared with sterile distilled water and the explants were soaked in the solution for 30 minutes. The explants were rinsed once in 40 mL of sterile distilled water followed by a 70% (v/v) ethanol wash for 2 minutes. The explants were then washed three (3) times with 40 mL of sterile distilled water for 5 minutes each and blotted on sterile filter paper (Whatman No. 1, 9 cm; Whatman plc, United Kingdom [UK]). The sterilised explants were cut into 1 cm^2 segments and inoculated into agar slants in vials containing full strength Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose, 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and solidified with 2.8 g/L Gelrite^{TM} (DUCHEFA, Netherlands) (Rosli *et al.*, 2009).

Callus induction was also carried out using leaf explants obtained from *in vitro* shoot cultures kindly provided by Felda Agricultural Services Sdn Bhd (FAS), Nilai, Negeri Sembilan. Leaf explants were aseptically separated from the stems and placed on the medium as stated above. All the explants used in callus induction were incubated in dark at 25 ± 2°C and callus cultures were maintained by sub-culturing at an interval of four weeks.

3.1.2 Induction and multiplication of somatic embryos

Embryogenic callus cultures provided by FAS were initiated aseptically into somatic embryos by culturing in 150 mL Erlenmeyer flasks containing full strength MS liquid medium supplemented with 3% (w/v) sucrose, 1.0 mg/L 2,4-D and 0.5 mg/L kinetin (Sobri *et al.*, 2005a). Liquid cultures were shaken at 120 rpm on orbital shaker (Wise Shake, Korea). All flasks were incubated at 25 ± 2°C under 16 hours photoperiod using cool white fluorescent lamps (Phillips TLD, 36W, 150 µmol.m^{-2}.s^{-1}). Somatic embryos from the initiation step were multiplied in full strength MS medium added with 3% (w/v) sucrose, 1.0 mg/L 2,4-D, 0.5 mg/L kinetin, 0.1% (w/v) activated charcoal and solidified with 2.8 g/L Gelrite™ (Sobri *et al.*, 2005a). Medium pH (Cyberscan PC 510 pH/mV/Conductivity/TDS/°C/°F Bench Meter, Eutech Instruments, Singapore) was adjusted to 5.8 after the addition of activated charcoal and prior to autoclaving (STURDY SA-300VFA-F-A505, Sturdy Industrial Co. Ltd., Taiwan). The embryos were incubated at 25 ± 2°C under 16 hours photoperiod using cool white fluorescent lamps (Phillips TLD, 36W, 150 µmol.m^{-2}.s^{-1}).

3.1.3 Propagation of *in vitro* shoot cultures

In vitro shoot cultures provided by FAS were cultured in full strength MS medium containing 3% (w/v) sucrose, 5.0 mg/L kinetin and solidified with 2.8 g/L Gelrite^{TM} (Sobri *et al.*, 2005b). The shoot cultures were incubated at 25 ± 2°C under 16 hours photoperiod using cool white fluorescent lamps (Phillips TLD, 36W, 150 µmol.m^{-2}.s^{-1}).

3.1.4 Surface sterilisation of seeds and growth of *in vitro* plantlets

Fresh *Eurycoma longifolia* fruits were rinsed with tap water to remove soil and other contaminants. The soft pericarp layer surrounding the seed coat was removed using forceps and scalpel and washed under running tap water with three (3) drops of Tween-20 (Sigma). The seeds were soaked in 50% (v/v) commercial Clorox solution and shaken vigorously for 30 minutes. Subsequent steps were carried out under aseptic conditions in laminar flow chamber. The seeds were rinsed three (3) times in 200 mL of sterile distilled water and blotted on sterile filter paper (Whatman No. 1, 9 cm; Whatman plc, United Kingdom [UK]). Cotyledons were removed from within the seed coat and placed on full strength MS medium with 3% (w/v) sucrose and solidified with 2.8 g/L Gelrite^{TM} (Murashige and Skoog, 1962) in Petri plates (BRANDON, 9cm). The cotyledons were germinated at 25 ± 2°C under 16 hours photoperiod using cool white fluorescent lamps (Phillips TLD, 36W, 150 µmol.m^{-2}.s^{-1}).

3.2 Microscopy observations of somatic embryos

3.2.1 Sample preparations for histological analysis

Freshly harvested embryo samples were cut diagonally into the sizes of 2-5 mm with a sharp knife. The tissue was then fixed in vials containing FAA [70% ethanol: glacial acetic acid: formaldehyde at the ratio of 18:1:1 (v/v/v)] at room temperature for 2 days and thoroughly washed with water every 2 hours, 1 day before beginning the dehydration process. During dehydration, fixed samples were washed using a graded series of alcohol with varying concentrations of 50, 70, 85, 98 and 100% (v/v) at room temperature. The embryos were then subjected to tertiary-butyl alcohol (TBA) wash I and II followed by immersion in xylene with wax I, II and III. Wax absorption is followed by embedding using a tissue embedding machine (Leica EG1160) at 61°C. Samples that were incubated in wax overnight were embedded at the center of a mold containing hot paraffin wax and allowed to cool to solidify. Solidified paraffin block were detached from the embedding mold and blocking was performed. Samples were dissected at thickness of 11 µM using a microtome (Leica RM2135). The specimen slides were then washed in histoclear solutions I and II continued by a graded series of alcohol at decreasing concentrations (100%, 90%, 70% and 50%) for 2 minutes each. The slides were left immersed for 24 hours in safranin stain (dissolved in 50% ethyl alcohol). To remove the safranin stain, the slides were washed with 70% and 80% alcohol (1 minute each) before immersion in fast green (dissolved in 95% ethyl alcohol) for 20 minutes. The stain was removed by washing the slides twice in 95% alcohol for 1 minute each followed by transfer to 100% alcohol (2 minutes) and histoclear III and IV (3

minutes each). Stained slides are allowed to air dry and mounted with cover slips. Samples were viewed using clinical laboratory microscope (Olympus BX41) and images were captured using Xcam-α.

3.2.2 Scanning electron microscopy (SEM) observations

To view the external structure of the micropropagated somatic embryos, freshly harvested embryos were used as samples in SEM studies. Two different techniques of sample preparation were utilised for observing somatic embryos of *Eurycoma longifolia*. Variable pressure scanning electron microscopy (VP SEM), also known as environmental scanning electron microscopy (ESEM) was carried out on the samples. The fresh embryo samples were removed from the multiplication medium, blotted on a filter paper and fixed on double-sided tapes that were affixed on the sample stub. Samples were neither treated nor coated prior to the viewing and were observed in their native state.

The second technique bears resemblance to conventional methods of sample preparations for SEM but without the inclusion of the dehydration steps. Embryo samples were washed at room temperature in distilled water, blotted dry on a filter paper and fixed on double-sided tapes that were affixed on the sample stub. The sample was transferred into the fume hood for fixing with 1% osmium tetroxide for 45 minutes prior to the viewing. Two somatic embryo samples were used for each technique of sample preparation and the observation was repeated once. All samples were viewed using the Leo Supra 50VP Field Emission SEM (Carl Zeiss SMT, Oberkochen, Germany).

3.3 *Agrobacterium rhizogenes* chemotaxis and attachment to somatic embryos

3.3.1 Preparation of *Agrobacterium rhizogenes* strains

Four strains of *Agrobacterium rhizogenes* were compared for their chemotactic ability. All strains were kindly provided by Dr. Lene H. Madsen from the University of Aarhus in Denmark. A4RS, an agropine strain harbouring pBIN19 plasmid containing kanamycin (*Kan*) resistance gene were streaked on Luria Bertani (LB) semi solid medium (Miller HiMedia, India) added with 50 mg/L kanamycin monosulfate (Duchefa, Netherlands) following the protocol by Subramaniam *et al.*, 2009 with slight modifications to the optical density (OD_{600nm}) of the culture. The *Agrobacterium rhizogenes* strains AR12, AR14 and AR1193 contain pBR322 plasmid sequences within the T_L-DNA and harbour rifampicin resistance gene for selection. These strains were streaked on Luria Bertani (LB) semi solid medium (Miller HiMedia, India) added with 100 mg/L rifampicin (Duchefa, Netherlands). The bacterial culture were incubated overnight in the dark at 26 ± 2°C for 2 days or until single colonies appear on the surface. A single colony of *Agrobacterium rhizogenes* on the semi solid medium were scooped out with a sterile wire loop and suspended in 30 mL LB broth medium (Miller HiMedia, India) containing their respective selection antibiotics. Broth cultures were shaken overnight in dark at 120 rpm on orbital shaker (Wise Shake, Korea) at 28 ± 2°C. The optical density (OD) of the culture at 600 nm was adjusted to 0.5-0.6 using spectrophotometer (Hitachi U-1900 UV/VIS, Japan) before preparing 1 mL working stocks containing 70% (v/v)

glycerol. These working stocks were revived in 30 mL LB broth medium containing selection antibiotics for use in chemotaxis and SEM attachment studies.

3.3.2 Medium preparation and protocol for chemotaxis assay

Modified swarm agar plate method was used to conduct chemotaxis assays (Shaw, 1995). The chemotactic medium contained 10 mM potassium phosphate buffer (pH 7.0), 1 mM ammonium sulfate, 1 mM magnesium sulfate, 0.1 mM potassium-EDTA, topped up with distilled water and solidified with 0.2% (w/v) Bacteriological agar (HiMedia, India).The medium was autoclaved at 121°C and 15psi for 15 minutes, poured into Petri plates (Brandon, 9 cm) and allowed to solidify. The bacterial strains were inoculated in the middle of the Petri plates using sterile 0.1 mL pipette tips.

Three (3) somatic embryos that were 4-6 mm in size were selected and cultured at a distance of 2.5 cm from the inoculation point. Embryos were either wounded by transverse cutting or unwounded. Chemotaxis was quantified after 72 and 96 hours of incubation at 24 ± 0.2°C in the dark. Control plate was prepared by inoculating the bacteria in the middle of the chemotactic medium without plant sample. The distance of bacterial movement, both towards and backwards from the point of inoculation was measured and used to obtain a ratio (R) using the following formula:

$$\text{Chemotaxis Ratio (Unit)} = \text{Towards/Backwards}$$

Towards is the distance of bacterial movement towards the explants exudates and backwards is the distance of bacterial movement away from the explants exudates. Each treatment consisted of ten (10) replicates containing three (3) somatic embryos each and was repeated once. Data collected were analysed for variation using two-way ANOVA and the differences were contrasted using Games-Howell's test at 5% significance level using IBM SPSS Statistics 20 (SPSS Inc. USA).

3.3.3 SEM observations of bacterial attachment

Working stocks of *Agrobacterium rhizogenes* strains A4RS, AR12, AR14 and AR1193 prepared with 70% (v/v) glycerol were revived by inoculating 1 mL of stock in 30 mL LB broth medium with their respective selection antibiotics. Broth cultures were shaken overnight in dark at 120 rpm on orbital shaker (Wise Shake, Korea) at 28 ± 2°C. The optical density (OD) of the culture at 600 nm was adjusted to 0.5-0.6 using spectrophotometer (Hitachi U-1900 UV/VIS, Japan). Somatic embryo explants, 4-6 mm in size were selected from four week old cultures grown in embryo multiplication medium. Explants were immersed in broth cultures for 20 minutes, blotted dry on sterile filter paper (Whatman No. 1, 9 cm; Whatman plc, United Kingdom [UK]) and inoculated on full strength MS medium with 3% (w/v) sucrose and solidified with 2.8 g/L Gelrite^{TM} (Murashige and Skoog, 1962). Explants were co-cultivated with *Agrobacterium rhizogenes* strains for 6 days in the dark at 25 ± 2°C. Four (2) co-cultivated explants were blotted to remove any gel attachments and placed in a Petri dish with a wet filter paper to ensure that the explants stay moist. The explants were then transferred into the fume hood for fixing with 1%

osmium tetroxide for one hour. The SEM sample preparation was done using the lyophilisation (freeze-drying) technique. Fixed samples were freeze dried, mounted onto a SEM specimen stub with a double sided sticky tape. The specimens were then coated with gold and viewed using Leo Supra 50VP Field Emission SEM (Carl Zeiss SMT, Oberkochen, Germany).

3.4 *Agrobacterium rhizogenes*-mediated transformation of *Eurycoma longifolia*

3.4.1 Plant materials

3.4.1.1 *In vivo* leaves and rachis

In vivo leaf and rachis explants were collected from *Eurycoma longifolia* plant grown at the School of Biological Sciences, Universiti Sains Malaysia (USM), Pulau Pinang. The explants were washed under running tap water and soaked in three to four drops of Tween-20 (Sigma) for 30 minutes. Subsequent steps were carried out in laminar flow chamber under aseptic conditions. Three (3) drops of Tween-20 were added as surfactant into 30% (v/v) commercial Clorox solution prepared with sterile distilled water and the explants were soaked in the solution for 30 minutes. The explants were rinsed once in 40 mL of sterile distilled water followed by a 70% (v/v) ethanol wash for 2 minutes. The explants were then washed three (3) times with 40 ml of sterile distilled water for 5 minutes each and blotted on sterile filter paper (Whatman No. 1, 9 cm; Whatman plc, United Kingdom [UK]). The sterilised leaf

explants were cut into 1 cm^2 segments and the rachis were cut at the length of 1 cm to prepare them for the hairy root induction experiment.

3.4.1.2 *In vitro* leaves, stems, roots and somatic embryos

The *in vitro* leaves and stems were obtained from two sources. The first source is the shoot cultures provided by FAS and grown in full strength MS medium containing 3% (w/v) sucrose, 5.0 mg/L kinetin and solidified with 2.8 g/L Gelrite™ (Sobri *et al.*, 2005b). The second are the seeds that have been surface sterilised and germinated into *in vitro* plantlets on full strength MS medium with 3% sucrose and solidified with 2.8 g/L Gelrite™ (Murashige and Skoog, 1962). The leaves were aseptically separated from the plantlets and the stems were cut into 0.8 mm segments to prepare them for the hairy root induction experiments. After the removal of the stem and leaves from the *in vitro* plantlets, the remaining intact root portion was used for the transformation experiment. For the hairy root induction experiments, three (3) leaves, three (3) stems and three (3) root portions were utilised per concentration of the auxins, indole-butyric acid (IBA) and indole-3-acetic acid (IAA) at the concentrations of 0, 0.5 and 1.0 mg/L. Four (4) to five (5) week old somatic embryos multiplied in full strength MS medium with 3% (w/v) sucrose, 1.0 mg/L 2, 4-D, 0.5 mg/L kinetin, 0.1% activated charcoal and solidified with 2.8 g/L Gelrite™ (Hussein *et al.*, 2005) were used for transformation.

3.4.2 Bacterial preparation for transformation

Agrobacterium rhizogenes strains A4RS, AR12, AR14 and AR1193 were revived by adding 1 mL of 70% glycerol working stock and 50 mg/L kanamycin (A4RS) and 100 mg/L rifampicin (AR12, AR14 and AR1193) respectively into 30 mL LB broth mediums. Broth cultures were shaken overnight at 120 rpm under dark conditions at 25 ± 2°C. A single loop of the revived broth culture was streaked on LB semi solid medium containing selection antibiotics to obtain single colonies. The single colony was added into LB broth medium containing selection antibiotics and shaken at 120 rpm in dark for 18 hours at 25 ± 2°C. The optical density (OD) of the culture at 600 nm was adjusted to 0.5-0.6 using spectrophotometer and 100 µM of acetosyringone was added to the culture prior to transformation (Subramaniam *et al.*, 2009).

3.4.3 Hairy roots induction from *in vivo* leaves, rachis and *in vitro* leaves, stems and roots of *Eurycoma longifolia*

To induce hairy roots in the *in vivo* leaves, rachis and *in vitro* leaves, stems and roots of *Eurycoma longifolia*, *Agrobacterium rhizogenes* cultures were grown for 18 hours, adjusted to an OD_{600nm} of 0.5-0.6 and added with 100 µM of acetosyringone, an inducer of virulence genes. The culture was drawn into a sterile fine needle syringe and a few drops were injected randomly into the leaf and root explants at a maximum of three (3) different regions. The culture was applied at the

cut surfaces of the rachis and stem explants. Explants were blotted off using sterile filter paper (Whatman No. 1, 9 cm; Whatman plc, United Kingdom [UK]) to remove excess bacterial suspension and co-cultivated in full strength Murashige and Skoog (MS) (1962) semi solid medium containing 3% (w/v) sucrose, 2.8 g/L Gelrite^{TM} and the auxins, indole-butyric acid (IBA) or indole-3-acetic acid (IAA) at the concentrations of 0, 0.5 and 1.0 mg/L. For the hairy root induction experiments from *in vivo* leaves and rachis, thirty (30) explants each were used and the experiment was repeated twice. For the *in vitro* leaves, stem and roots, three (3) explants each were utilised per concentration of the auxins, IBA and IAA and were repeated once. Co-cultivation was conducted at 24 ± 0.2°C under dark conditions in an incubator for 3 days. Explants were then washed with 500 mg/L cefotaxime sodium and subsequently transferred into fresh co-cultivation medium until the excess bacteria were eliminated. For negative control, the explants were subjected to the same treatment as above except that the explants were inoculated with blank LB broth medium.

3.4.4 Optimisation of transformation efficiency in *Eurycoma longifolia* somatic embryos

3.4.4.1 Experimental design: Optimisation of parameters

To assess factors affecting the transformation frequency, four parameters were compared for each factor on transformation efficiency. A range of parameters were evaluated and for each parameter, three (3) replicates were used containing ten (10) somatic embryos per replicate and were repeated three (3) times. Parameters

included virulence of *Agrobacterium rhizogenes* strains (A4RS, AR12, AR14 and AR1193), influence of indole-butyric acid (IBA) [0, 0.5, 1.0, 1.5 and 2.0 mg/L] and different concentrations of the antioxidants, dithiothreitol (DTT) [0, 1, 2, 3 and 4 mg/L] and polyvinylpyrrolidone (PVP) [0, 1, 2, 3, and 4% (w/v)] in full strength MS medium supplemented with 3% (w/v) sucrose and 2.8 g/L Gelrite™.

Somatic embryos, 4-6mm in size, were first pre-cultured for two (2) days in full strength MS medium containing 0.5 mg/L IBA, 2.0 mg/L DTT, 1% (w/v) PVP, 3% (w/v) sucrose and solidified with 2.8 g/L Gelrite™. *Agrobacterium rhizogenes* cultures which have been grown for 18 hours were adjusted to an OD_{600nm} of 0.5-0.6 and added with 100 µM acetosyringone were used for transformation. The embryo explants were cut in half lengthwise and immersed in the bacterial suspension for 20 minutes. The explants were then blotted with sterile filter paper (Whatman No. 1, 9cm; Whatman plc, United Kingdom [UK]) and placed back in the medium for co-cultivation in the dark for 3 days at 24 ± 0.2°C. Subsequently, co-cultivated explants were washed in full strength MS broth medium containing 3% (w/v) sucrose and 500 mg/L cefotaxime sodium and transferred into fresh co-cultivation medium for four (4) days in the dark at 24 ± 0.2°C. One factor of the standard conditions was changed each time and the effects on percentage of transient *gusA* gene expression were evaluated to determine the optimum conditions for transformation,.

3.4.4.2 GUS histochemical assay

The transient expression levels of *gusA* gene in the somatic embryos were assayed and compared 7 days after co-cultivation. GUS activity was localized

histochemically as described previously by Jefferson (1987). A modified GUS protocol developed for the identification of transformed roots of coffee plants (Alpizar *et al.*, 2006) were used to assay for *gusA* activity in the somatic embryo explants. Explants were washed in full strength MS broth medium containing 3% (w/v) sucrose and 500 mg/L cefotaxime sodium to eliminate excess bacteria and immersed in a staining solution containing 1 mM 5-bromo-4-chloro-3-indolyl-β-d-glucuronide (X-Gluc), 50 mM disodium hydrogen phosphate (Na_2HPO_4, pH 7.0), 0.5 mM potassium ferricyanide [$K_3Fe(CN)_6$)] and 0.5 mM potassium ferrocyanide [$K_4Fe(CN)_6.3H_2O$] in 24-well microwell plates and incubated for 36 to 40 hours at 37°C. After staining, the explants were washed with 70% ethanol twice and treated with FAA [70% ethanol: glacial acetic acid: formaldehyde at the ratio of 18:1:1 (v/v/v)] for 2 days to remove chlorophyll before observation. A sample was scored as transient GUS positive if at least 25% of the embryo's surface exhibited blue-region. Data were analysed using one-way ANOVA and the differences contrasted using Games-Howell's test at 5% significance level using IBM SPSS Statistics 20 (SPSS Inc. USA).

3.4.4.3 Induction of hairy roots in somatic embryos of *Eurycoma longifolia*

The induction of hairy roots from somatic embryo explants was conducted following the transformation optimisation protocol. Somatic embryos, 4-6 mm in size, were first pre-cultured for two (2) days in full strength MS medium containing 1.0 mg/L IBA, 1% (w/v) PVP, 3% (w/v) sucrose and solidified with 2.8 g/L Gelrite™. *Agrobacterium rhizogenes* cultures which have been grown for 18 hours

were adjusted to an OD_{600nm} of 0.5-0.6 and added with 100 µM acetosyringone were used for transformation. The embryo explants were cut in half lengthwise and immersed in the bacterial suspension for 20 minutes. The explants were then blotted with sterile filter paper (Whatman No. 1, 9 cm; Whatman plc, United Kingdom [UK]) and placed back in the medium for co-cultivation in the dark for 3 days at 24 ± 0.2°C. Subsequently, co-cultivated explants were washed in full strength MS liquid medium containing 3% (w/v) sucrose and 500 mg/L cefotaxime sodium and transferred into root induction medium containing 1.0 mg/L IBA, 1% (w/v) PVP, 3% (w/v) sucrose, 1.0 g/L activated charcoal and solidified with 2.8 g/L Gelrite™. The embryos were incubated at 25 ± 2°C under 16 hours photoperiod using cool white fluorescent lamps (Phillips TLD, 36W, 150 $\mu mol.m^{-2}.s^{-1}$) and were observed for the formation of hairy roots. Each hairy root induction experiment consisted of ten (10) replicates containing ten (10) somatic embryos each and was repeated twice.

3.4.5 Genomic DNA extraction and detection of transformants using polymerase chain reaction (PCR)

Genomic DNA was extracted from the transformed and control somatic embryos using the Genomic DNA Mini Kit (Plant; Geneaid Biotech Ltd., Taipei County, Taiwan, Republic of China) following the manufacturer's instructions. The DNA purity and concentration was subsequently checked using a micro volume nucleic acid spectrophotometer (ACTGene ASP-2680). The purity of the genomic DNA sample extracted from the control and transformed somatic embryos was determined to be 1.8 and 1.9, respectively. The PCR experiments were performed

using sequences of *rolA*, *rolB*, *rolC* primers and the virulence gene primer, *virD*. The primer sequences are as follows:

- *rolA* primers - Forward: 5'-CGTTGTCGGAATGGCCCAGACC-3'
  Reverse: 5'-CGTAGGTCTGAATATTCCGGTCC-3'
- *rolB* primers - Forward: 5'-ATGGATCCCAAATTGCTATTCCTTCCACGA-3'
  Reverse: 5'-TTAGGCTTCTTTCTTCAGGTTTACTGCAGC-3'
- *rolC* primers – Forward: 5'-TGTGACAAGCAGCGATGAGC-3'
  Reverse: 5'-GATTGCAAACTTGCACTCGC-3'
- *virD* primers – Forward: 5'-ATGTCGCAAGGACGTAAGCCCA-3'
  Reverse: 5'-GGAGTCTTTCAGCATGGAGCAA-3'

The reaction mixture comprises of 5.0 ng of genomic DNA, 1X Dream Taq Buffer with 2 mM $MgCl_2$, 0.2 mM dNTPs, 1.5 U/µL of Dream Taq Polymerase and 0.2 µM of each primer and deionised water, added to make a final volume of 25 µL. For DNA amplification, samples were subjected to an initial denaturation step of 94°C/3 minutes, followed by 35 cycles of denaturation at 94°C/1 minute, an annealing step at 60.5°C/1 minute, extension at 72°C/1 minute and a final extension at 72°C for 10 minutes The amplified products were fractionated in 1.0% (w/v) agarose gel at 85V, stained with ethidium bromide and viewed under UV illuminator.

CHAPTER 4

RESULTS

4.1 Micropropagation of *Eurycoma longifolia*

4.1.1 Callus induction from *in vivo* and *in vitro* leaf explants

Callus induction was conducted on both *in vivo* and *in vitro* leaf explants to obtain suitable explants for transformation protocol. The leaf explants were routinely sub-cultured into fresh media at an interval of four (4) weeks. Observation on callus formation was carried out weekly to determine the time required for the induction process. Plate 4.1 shows the friable callus induced from *in vitro* leaf explants of *Eurycoma longifolia* using full strength MS medium supplemented with 2.0 mg/L 2,4-D. Beads of callus were observed on the surface of *in vitro* leaf explants cultured on callus induction medium three (3) weeks from incubation. However, surface sterilised *in vivo* explants cultured on the same medium developed callus after fourteen (14) weeks of incubation at 25 ± 2°C in the dark. It was noted that both types of leaf explants underwent browning during incubation. Callus formed by the *in vitro* explants were light yellow in colour, friable and fast growing. The surface sterilised explants yielded dark yellow friable callus that grew slowly and turned brown several weeks after the induction was recorded, despite the sub-culturing at regular intervals.

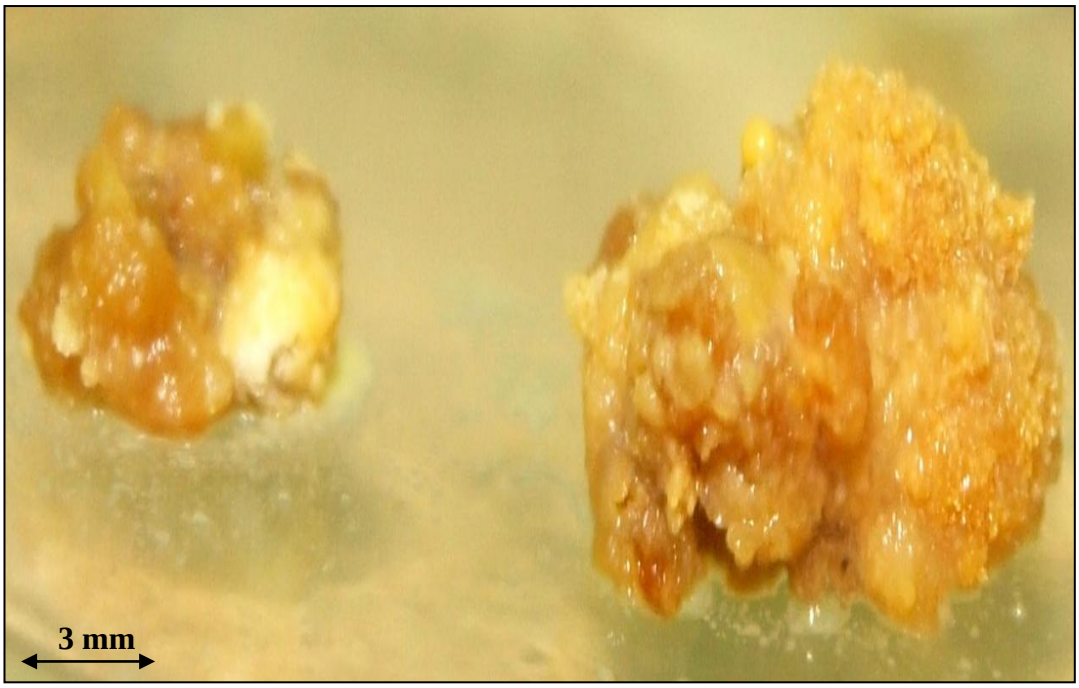

Plate 4.1: Friable callus induced from *in vitro* leaf explants of *Eurycoma longifolia* using full strength MS medium supplemented with 2.0 mg/L 2, 4-D.

4.1.2 Induction and multiplication of somatic embryos

Embryogenic callus cultures provided by FAS were initiated aseptically into somatic embryos by culturing in full strength MS liquid medium supplemented with 3% (w/v) sucrose, 1.0 mg/L 2,4-D and 0.5 mg/L kinetin. After three (3) weeks in shaken flask cultures incubated at 25 ± 2°C with 16 hour photoperiod, the callus were observed to have turned into green embryos from its initial yellow, friable callus state. These were then transferred into full strength MS semi solid medium containing 3% (w/v) sucrose, 1.0 mg/L 2,4-D and 0.5 mg/L kinetin and 1.0 g/L activated charcoal and propagated under the same physical conditions.

The observation indicates that somatic embryos of *Eurycoma longifolia* could not survive for prolonged periods of time in a broth medium. Embryos that were cultured for more than three (3) weeks in broth medium underwent yellowing and subsequent browning, losing its growth capacity in the process. Excessive injury to the embryos during the sub-culture resulted in browning as well. However when the somatic embryos were not injured, the capacity for the formation of new embryos were affected as seen by the lesser number of somatic embryos formed by uninjured embryos.

All the somatic embryo cultures were sub-cultured periodically at an interval of 4-5 weeks as the embryos underwent yellowing followed by dedifferentiation into embryogenic callus as the incubation period was prolonged. During the incubation, the somatic embryos exhibited all the stages of growth characteristic of zygotic embryos such as globular, heart and torpedo stages. Plate 4.2 shows somatic embryo cultures in embryo multiplication medium containing full strength MS basal salts

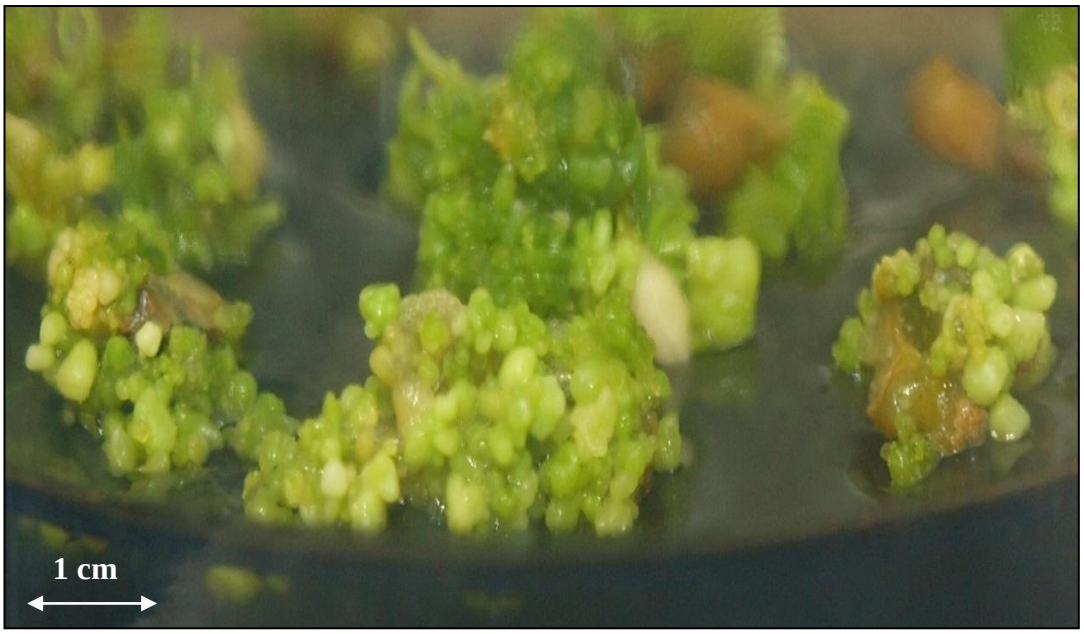

Plate 4.2: Somatic embryo cultures of *Eurycoma longifolia* propagated on full strength MS semi solid medium with 1.0 mg/L of 2,4-D, 0.5 mg/L kinetin and 1.0 g/L activated charcoal.

with 3% (w/v) sucrose, 1.0 mg/L 2,4-D, 0.5 mg/L kinetin, 1.0 g/L activated charcoal and solidified with 2.8 g/L Gelrite™.

4.1.3 Propagation of *in vitro* shoot cultures

Shoot cultures provided by FAS were grown in full strength MS semi solid medium containing 3% (w/v) sucrose and 5.0 mg/L kinetin. The shoots were sub-cultured in fresh medium at an interval of eight (8) weeks and were observed to grow slowly, requiring approximately three (3) months to achieve the growth shown in Plate 4.3.

4.1.4 Surface sterilisation of seeds and growth of *in vitro* plantlets

Cotyledons from surface sterilised seeds were removed from within the seed coat and germinated on full strength MS medium with 3% (w/v) sucrose and solidified with 2.8 g/L Gelrite™. Germination was achieved within two (2) weeks of culture in the medium, as the roots emerged first followed by the emergence of shoots. Widespread fungal contaminations were observed following the surface sterilisation, effectively preventing more seedlings from being germinated *in vitro*. Many of the seeds that were free of contamination failed to germinate after the surface sterilisation protocol, thus reducing the number of plantlets available for transformation further. The seeds that were able to germinate were observed to

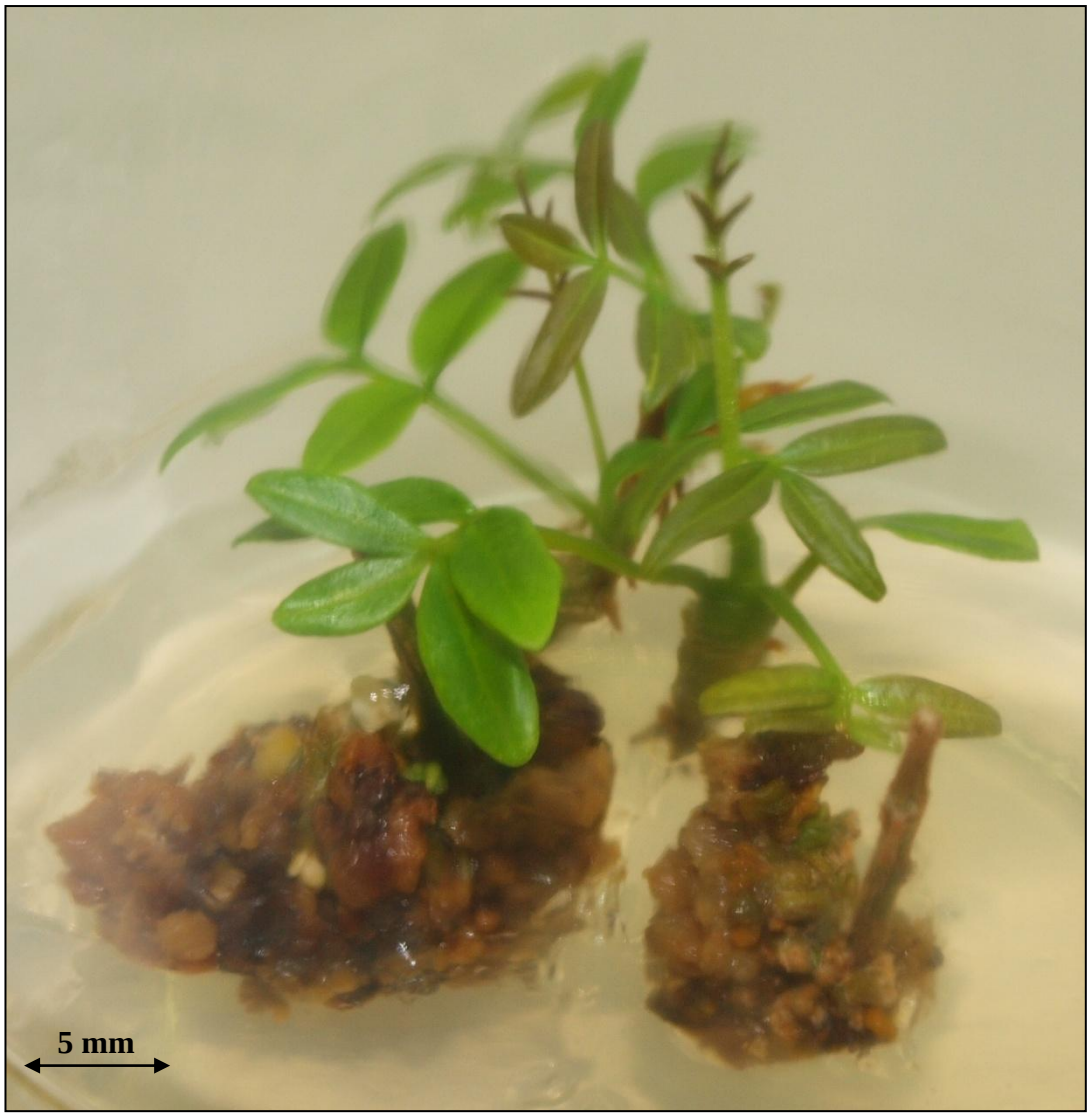

Plate 4.3: ***In vitro*** **shoot cultures grown in full strength MS semi solid medium containing 3% (w/v) sucrose and 5.0 mg/L kinetin.**

undergo the regular germination stages characteristic of zygotic embryos in the wild. Although the roots were seen emerging as early as two (2) weeks from culture, the shoots took a much longer time to emerge, appearing only after about three months, during which they were sub-cultured into full strength medium containing 5.0 mg/L kinetin. Plate 4.4 shows an *in vitro* plantlet germinated from surface sterilised seed of *Eurycoma longifolia*.

4.2 Microscopy observations of somatic embryos

4.2.1 Histological observations

Somatic embryos were multiplied in full strength MS broth medium supplemented with 3% (w/v) sucrose, 1.0 mg/L 2,4-D and 0.5 mg/L kinetin. The somatic embryos of *E. longifolia* were confirmed using histological analysis by examining the characteristic morphological changes. The cultured somatic embryos were bipolar in structure, possessing both plumular and radicular ends, thus closely resembling their zygotic counterpart. Both the shoot and root apical meristem includes densely arranged meristematic initials that could be seen in the darker regions of the embryo cross section. Plates 4.5 and 4.6 show the histological observation of shoot and root apex of *Eurycoma longifolia* somatic embryos.

Plate 4.4 *In vitro* plantlet grown from surface sterilised *Eurycoma longifolia* seeds and germinated in full strength MS semi solid medium.

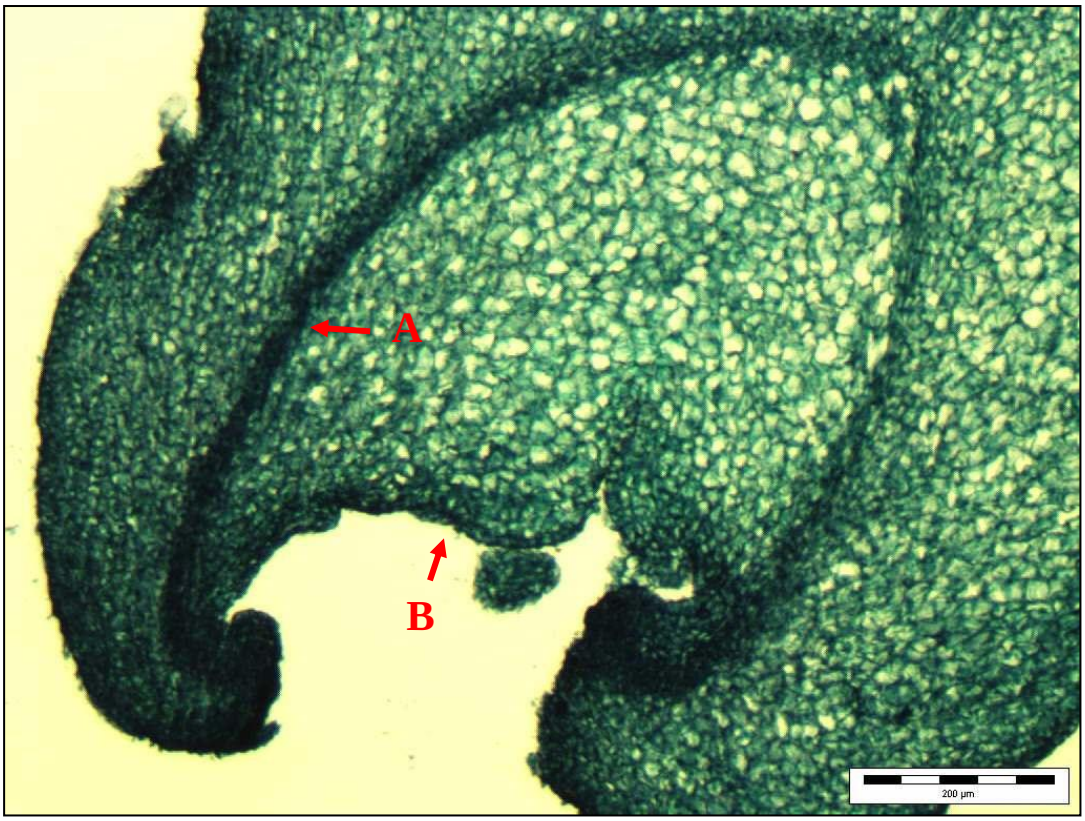

Plate 4.5: Histological observation of the shoot apex of *Eurycoma longifolia* somatic embryos. (A) Procambium vascular meristem **(B)** Shoot apical meristem.

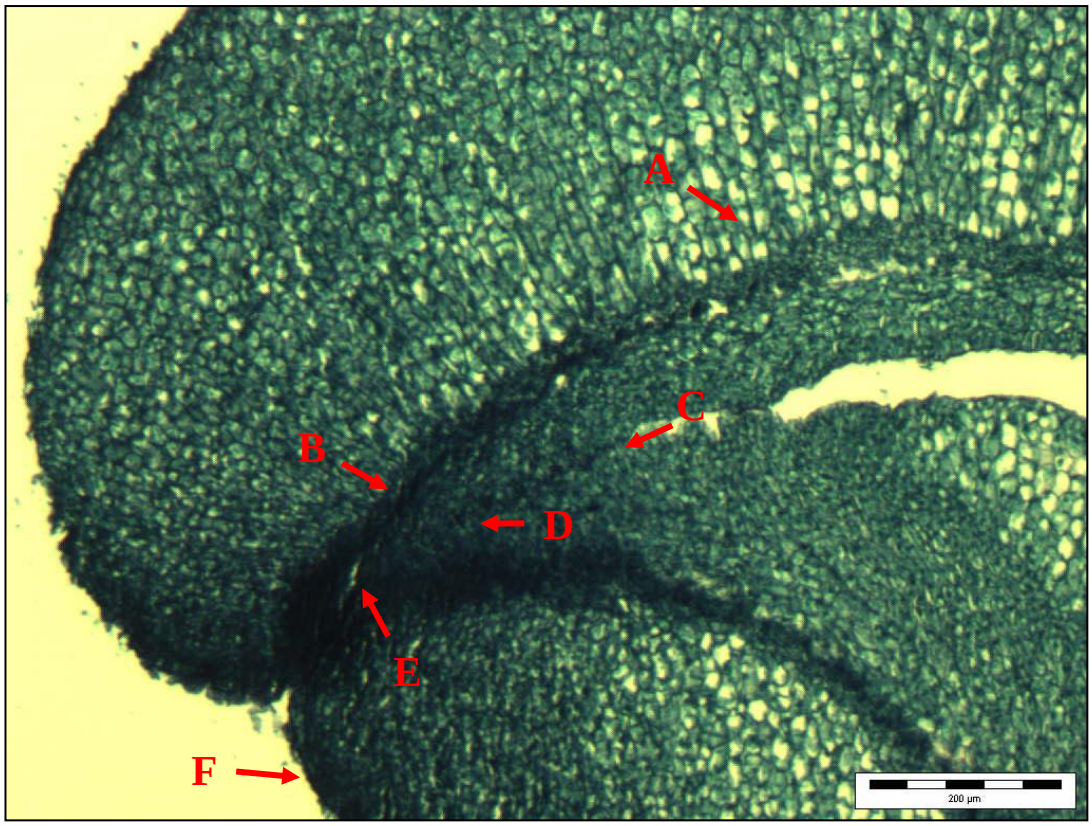

Plate 4.6: Histological observation of the root apex of *Eurycoma longifolia* somatic embryos. (A) Protoderm **(B)** Cortex **(C)** Central cylinder **(D)** Initials of central cylinder **(E)** Initials of root cap **(F)** Root cap.

4.2.2 SEM observations

The surface morphology of somatic embryos was observed using scanning electron microscope (SEM) and shown in Plates 4.7, 4.8 and 4.9. Somatic embryos were subjected to two different types of sample preparation prior to viewing. In the first, no sample preparation was conducted and the fresh samples were viewed in their native state using the ESEM method. Plate 4.7 is the image of two embryos with noticeable shoot and root apical meristems. Embryos could be seen emerging from the meristematic structure at the base. Prominent cotyledons were also observed on both embryos. Plate 4.8 shows a closer view of the epidermal surface of the embryo. The cells appear to be shrunken due to the loss of moisture and the intense pressure exerted by the technique. Plate 4.9 is the embryos viewed after fixing the sample with 1% osmium tetroxide. The cells could be seen maintaining their integrity despite the pressure. However, prolonged exposure does cause damage to the surface of the embryo.

4.3 Interaction between *Agrobacterium rhizogenes* and *Eurycoma longifolia*

4.3.1 Chemotaxis movement assay

Chemotaxis assays were carried out using the modified swarm agar plate method. Observations of *A. rhizogenes* strains in the swarm plates show that all the strains took approximately 48 hours for visible spread of the bacteria to appear on the surface of the medium. The gradient created by the presence of diffused chemicals or

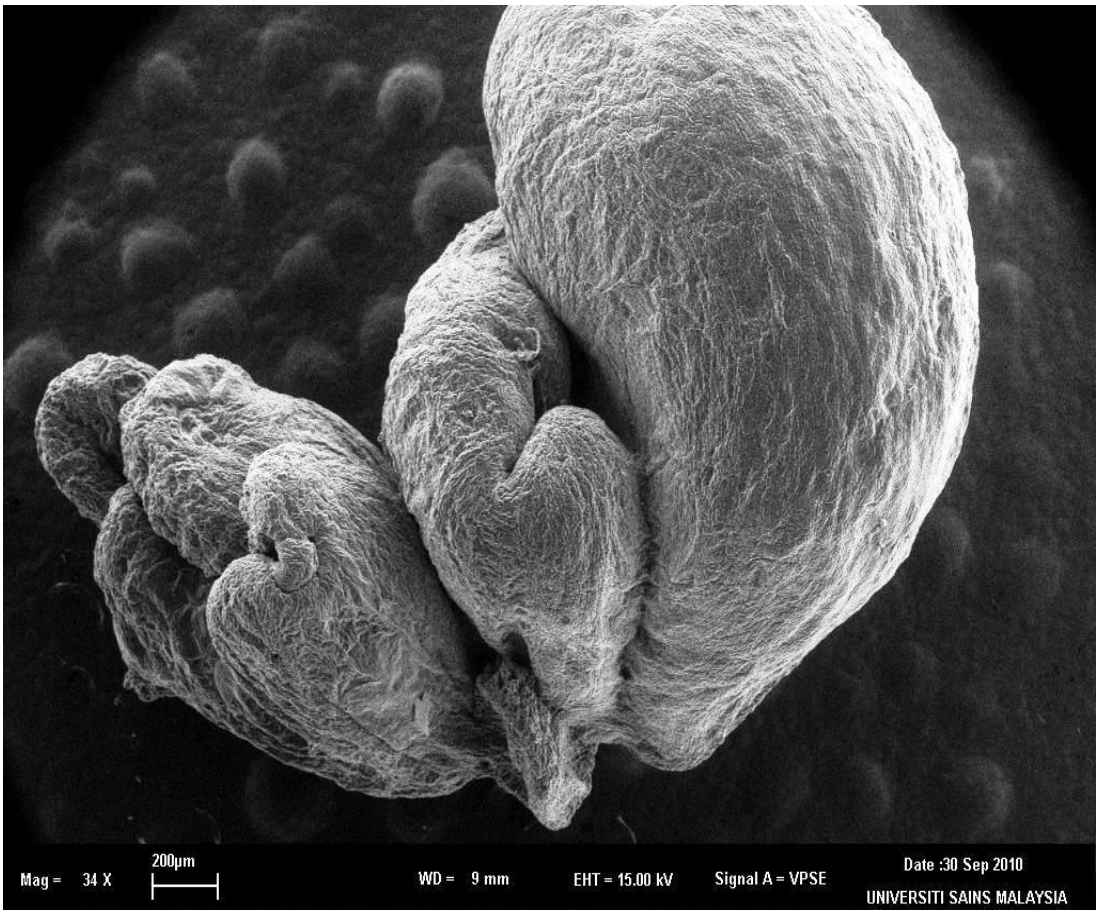

Plate 4.7: Scanning electron microscope (SEM) observation of *Eurycoma longifolia* somatic embryo surface using the environmental scanning electron microscopy (ESEM) method.

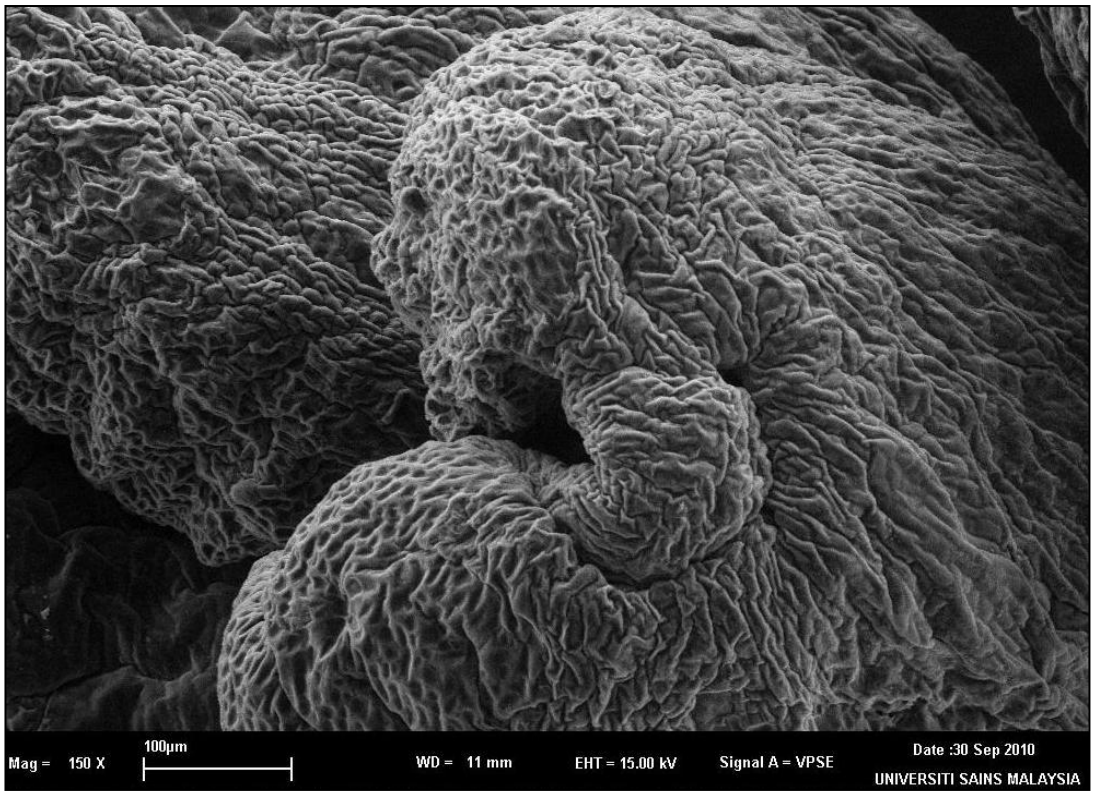

Plate 4.8: Scanning electron microscope (SEM) observation of *Eurycoma longifolia* somatic embryo surface using the environmental scanning electron microscopy (ESEM) method. The close up image of the surface shows shrunken cells that are caused by the method.

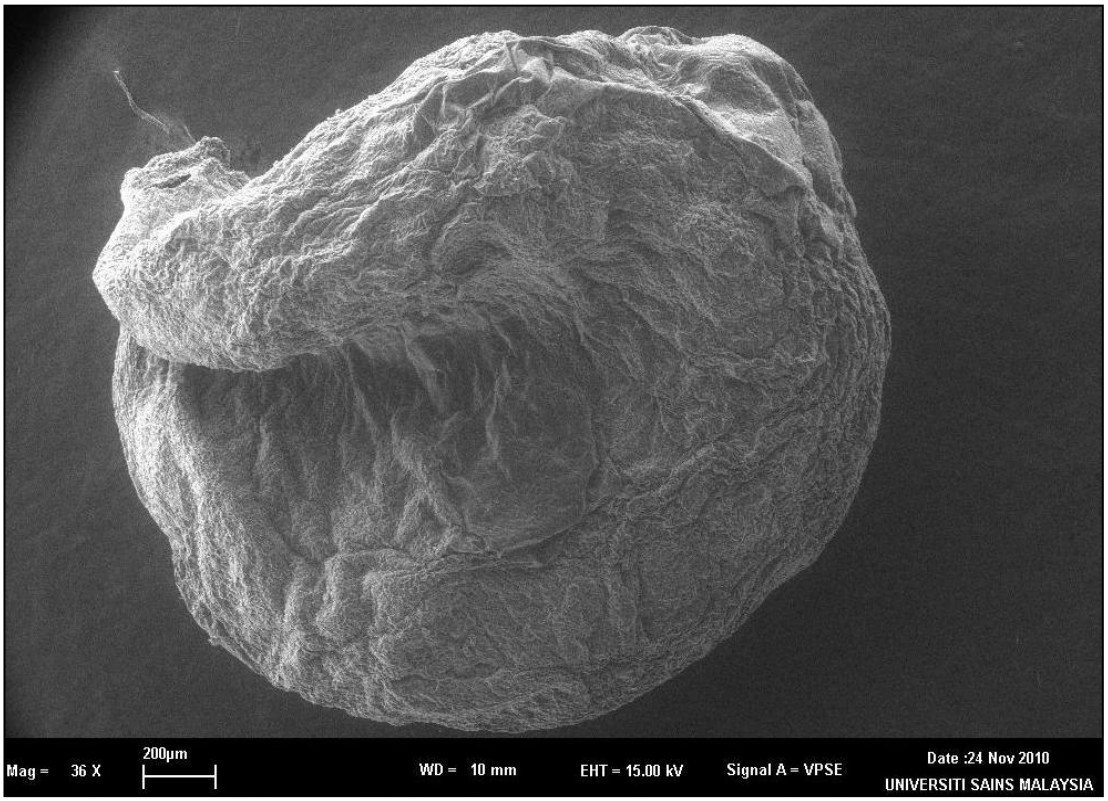

Plate 4.9: Scanning electron microscope (SEM) observation of *Eurycoma longifolia* somatic embryo surface after fixing with 1% osmium tetroxide. The surface shows cells that still maintain their structural integrity despite the pressure exerted by the microscope.

plant wound exudates at the edges of the plates acted as attractants for the bacteria incubated on the semi solid agar plates and swarming of bacteria outward from the central point of inoculation was observed. As the bacterial swarming was visible to the naked eye, it allowed us to quantify the chemotactic response of *A. rhizogenes*.

The chemotactic behaviour of *Agrobacterium* was found to be perpetually positive for all the bacterial strains tested, independently of the wounding level. The overall swarming ratio of the bacterial strains tested in the presence of somatic embryos ranged between 1.10 and 1.30, indicating a positive effect of the plant exudates on bacterial movement. This effect is demonstrated by the results obtained from the four strains (A4RS, AR12, AR14 and AR1193) after assaying the excised tissues (Figs. 4.1 and 4.2). The quantification of swarming ratios for A4RS strain proved more difficult compared to AR12, AR14 and AR1193 strains due to the lack of growth intensity in the swarm.

4.3.2 SEM observation of bacterial attachment

Scanning electron microscope (SEM) was used to view the surface of four (4) week old somatic embryo explants immersed in Luria-Bertani (LB) broth medium containing *A. rhizogenes* cells and co-cultivated for six (6) days in the dark at 25 ± 2°C. Sample preparation was carried out by fixing the sample in 1% osmium tetroxide, freeze-dried, coated with gold particles and mounted on a sample stub. The marked difference between the condition of the sample prepared using this technique and ESEM method was noted. Samples prepared using the freeze-drying technique was remarkably well preserved and did not deteriorate during the viewing process

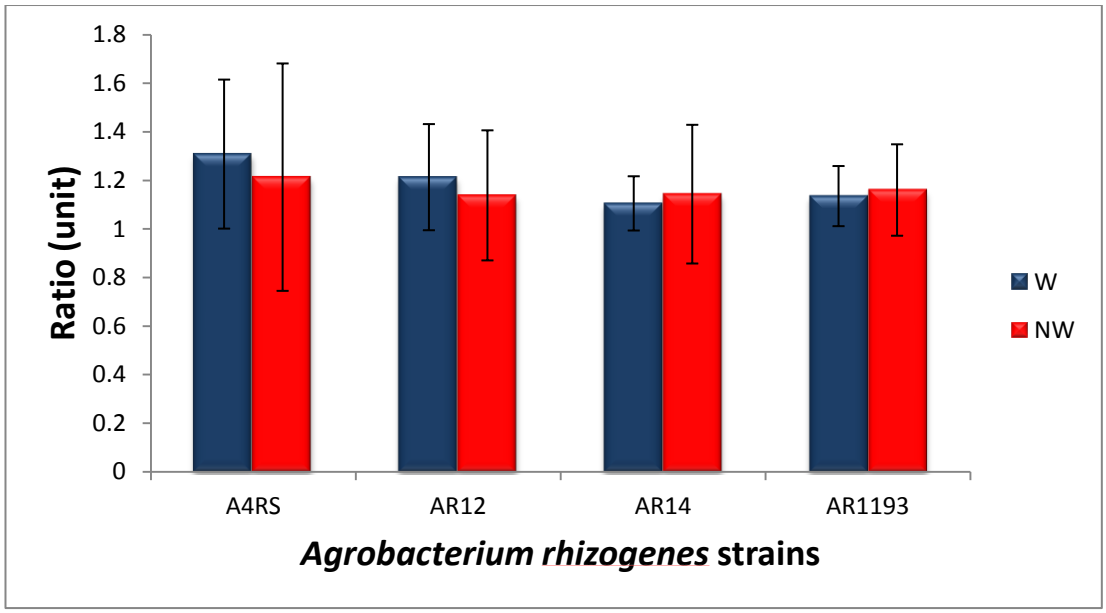

Figure 4.1: Chemotaxis assay of *Agrobacterium rhizogenes* towards wounded and non-wounded somatic embryo explants of *Eurycoma longifolia* after 72 hours. W = Wounded, NW = Not wounded. Error bars show standard deviation. Means in the experiment were analysed using two-way ANOVA and differentiated with Games-Howell's test (α=0.05).

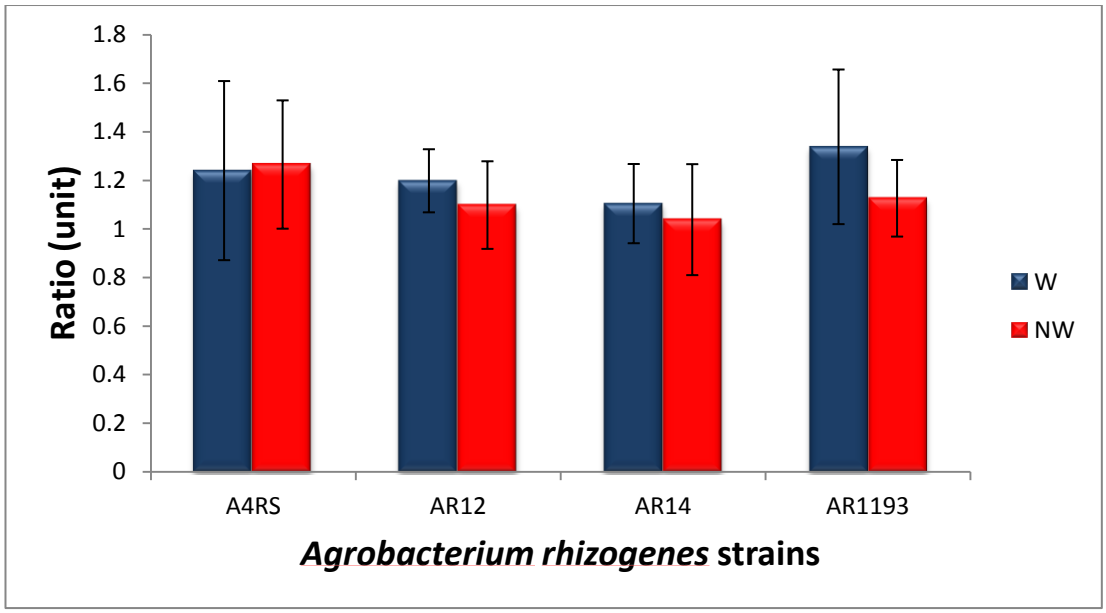

Figure 4.2: Chemotaxis assay of *Agrobacterium rhizogenes* towards wounded and non-wounded somatic embryo explants of *Eurycoma longifolia* after 96 hours. W = Wounded, NW = Not wounded. Error bars show standard deviation. Means in the experiment were analysed using two-way ANOVA and differentiated with Games-Howell's test (α=0.05).

Plate 4.10 (A) shows the surface of the embryo covered with a mesh-like layer of *A. rhizogenes* cells. While *A. rhizogenes* cells did attach to the exposed surfaces of the somatic embryo explants, their distribution was not uniform. Figure 4.10 (B) shows a close up of the embryo explants, where the formation of *Agrobacterium* biofilm over the surface becomes more evident. Figure 4.10 (C) shows the bacteria attached by fibrillar connections to each other and the rod-shaped cells are densely packed on the web surrounding the explants. The cells are not only attached to one another but to the cell surfaces as well, as observed in Figure 4.10 (D). Strands were seen running from the bacteria to the plant cell walls and these strands looked identical. There were one or several strands attaching each bacterial cell to the embryo explant. However, there were areas in all of these explants where the bacteria did not attach.

4.4 *Agrobacterium rhizogenes*-mediated transformation of *Eurycoma longifolia*

4.4.1 Hairy roots induction from *in vivo* leaves, rachis and *in vitro* leaves, stems and roots of *Eurycoma longifolia*

Various explants of *Eurycoma longifolia* such as *in vivo* leaves, *in vivo* rachis, *in vitro* leaves and *in vitro* roots failed to form hairy roots when the transformation process was conducted using the disarmed strains of *Agrobacterium rhizogenes*. *In vivo* leaves and rachis were surface sterilised and revealed a very high contamination rate, reaching up to 100% at times. The explants that were free from contamination turned brown and necrotic soon after the surface sterilisation and transformation protocol. No hairy root induction was achieved in both these explants. The injection

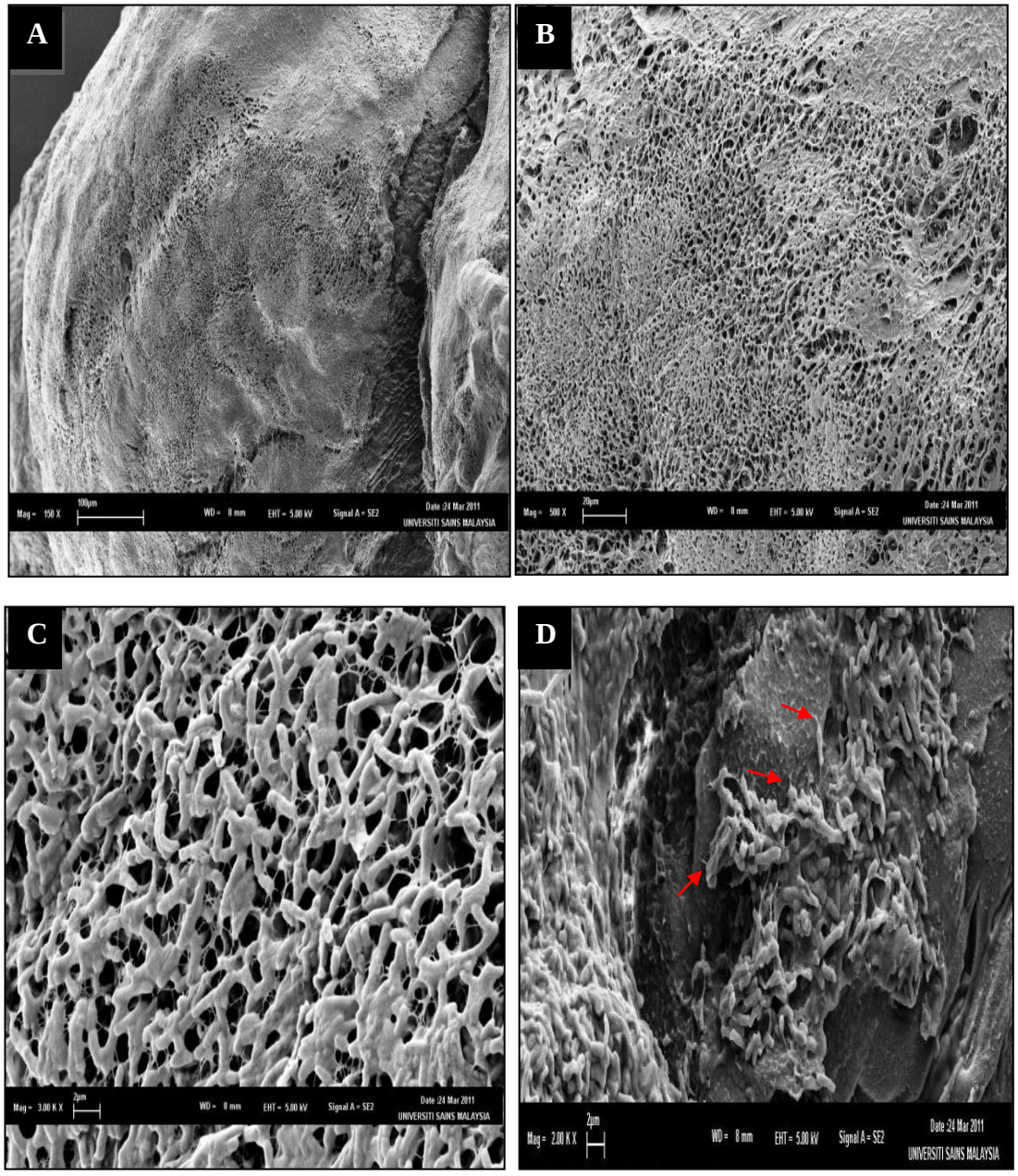

Plate 4.10: Scanning electron photomicrographs of attachment of *Agrobacterium rhizogenes* cells on the surface of *Eurycoma longifolia* somatic embryos. (A) An overview image of somatic embryo surface; **(B)** enlargement of panel A shows *A. rhizogenes* forming a biofilm on the surface of the embryos; **(C)** An enlargement of panel B shows a membranous formation of *A. rhizogenes* cells; **(D)** *A. rhizogenes* attachments on plant cell wall with bacterial attachment strands extending to the cells. Arrows show the bacterial strands attached to the somatic embryo.

method that was used for the *in vitro* explants caused an injury response as the injected regions were seen to be yellowing outward, followed by tissue browning and death. The leaf explants were the most fragile and did not survive the transformation protocol and the subsequent washes with cefotaxime followed by the root explants.

The stem explants however, did not undergo the process of tissue death despite the yellowing observed following the transformation protocol. The explants were washed in broth medium containing 500 mg/L cefotaxime sodium and transferred to mediums containing 0, 0.5 and 1.0 mg/L of IAA or IBA. While no roots were observed from any of the stem explants incubated in IAA medium, three stem explants transformed with *Agrobacterium rhizogenes* strain AR14 and co-cultivated in 0.5 mg/L IBA produced hairy root-like structures. No rooting was observed with any of the other *A. rhizogenes* strains used for transformation. It was noted that the rooting was achieved at the base of the stem explant, where it comes in contact with the auxin-containing medium. The stem was first observed to form a callus tissue at the base followed by the emergence of root initials as seen in Plates 4.11 and 4.12. Various small hairy root-like protrusions were seen emerging from the stem explant and noted to be growing outward and upward, instead of towards the medium, indicating that these are possibly non-geotropic in nature. The fragile, brittle roots were white yellowish in colour and brown at the base.

Plate 4.11: Frontal view of transformed *Eurycoma longifolia* stem with hairy root like structure seen proliferating from the cut base of the stem. Explants were co-cultivated with *Agrobacterium rhizogenes* strain AR14 in full strength MS medium with 0.5 mg/L indole-butyric acid (IBA).

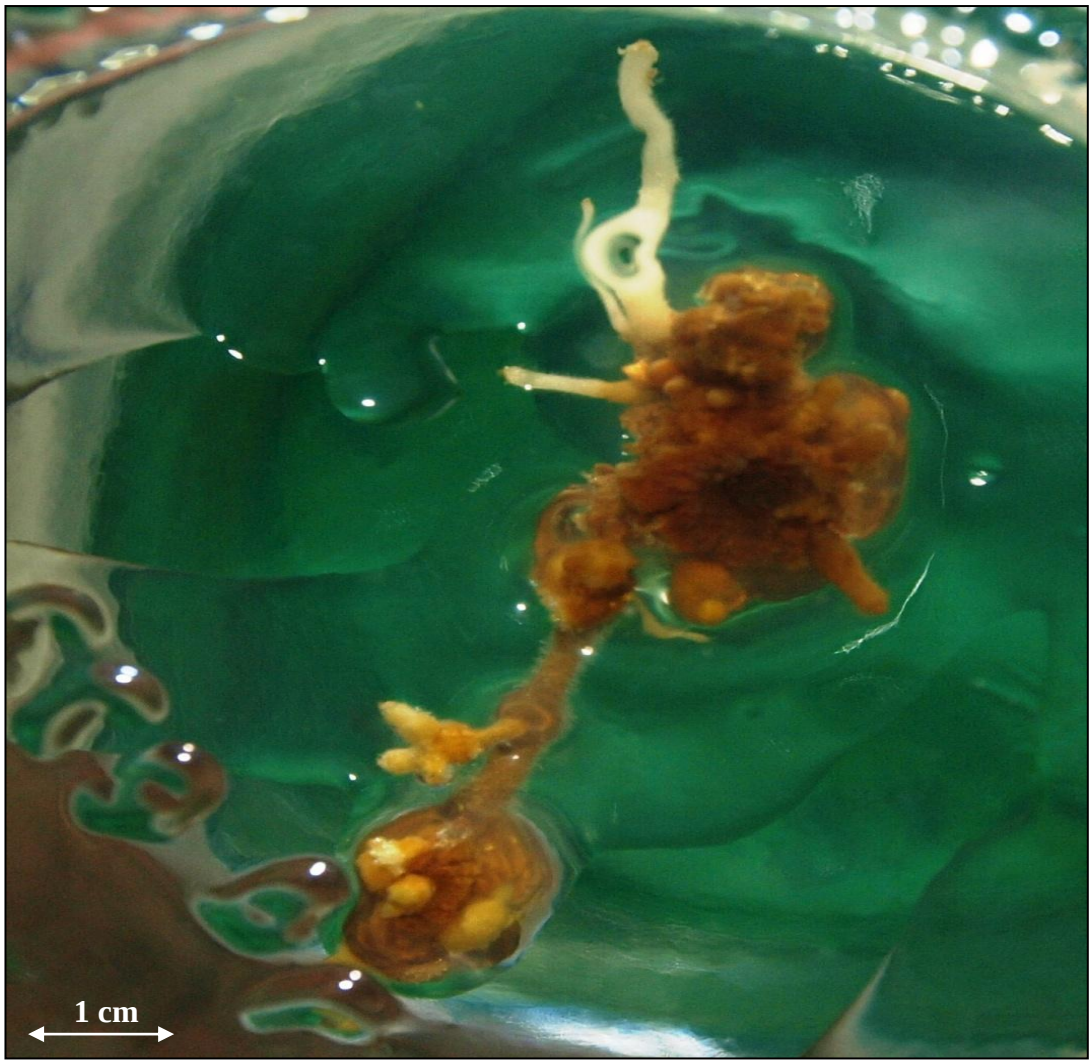

Plate 4.12: Bottom view of transformed *Eurycoma longifolia* stem with multiple hairy root-like protrusions seen proliferating from the cut base of the stem. Explants were co-cultivated with *Agrobacterium rhizogenes* strain AR14 in full strength MS medium with 0.5 mg/L indole-butyric acid (IBA).

4.5 Optimisation of transformation efficiency in *Eurycoma longifolia* somatic embryos

4.5.1 Virulence of *Agrobacterium rhizogenes* strains

The highest percentage of GUS positive explants were observed for *A. rhizogenes* strain AR12 at 84.2% followed by AR14 (68.3%), AR1193 (55.0%) and A4RS (44.2%) as shown in Figure 4.3. The explants that were used for the negative controls did not exhibit any GUS positive results. It was noted that there are no significant differences between the GUS positive results observed for AR12 and AR14, between AR14 and AR1193 and between AR1193 and A4RS.

Visual observation of the explants transformed with the various strains of *A. rhizogenes* did not exhibit any marked difference in the intensity of the GUS staining as shown in Plate 4.13. The criteria used to determine GUS postitive explants were based on the appearance of blue stain on more than 25% of the explant surface. It was noted that explants transformed with AR12 exhibited the highest coverage of the surface as many showed coverage of up to 75%. These were not observed in the explants transformed with *A. rhizogenes* strains AR14, AR1193 and A4RS. Due to these observations coupled with the high GUS positive percentage shown by AR12, this strain was chosen for the subsequent transformation optimisation step using various concentrations of the hormone IBA.

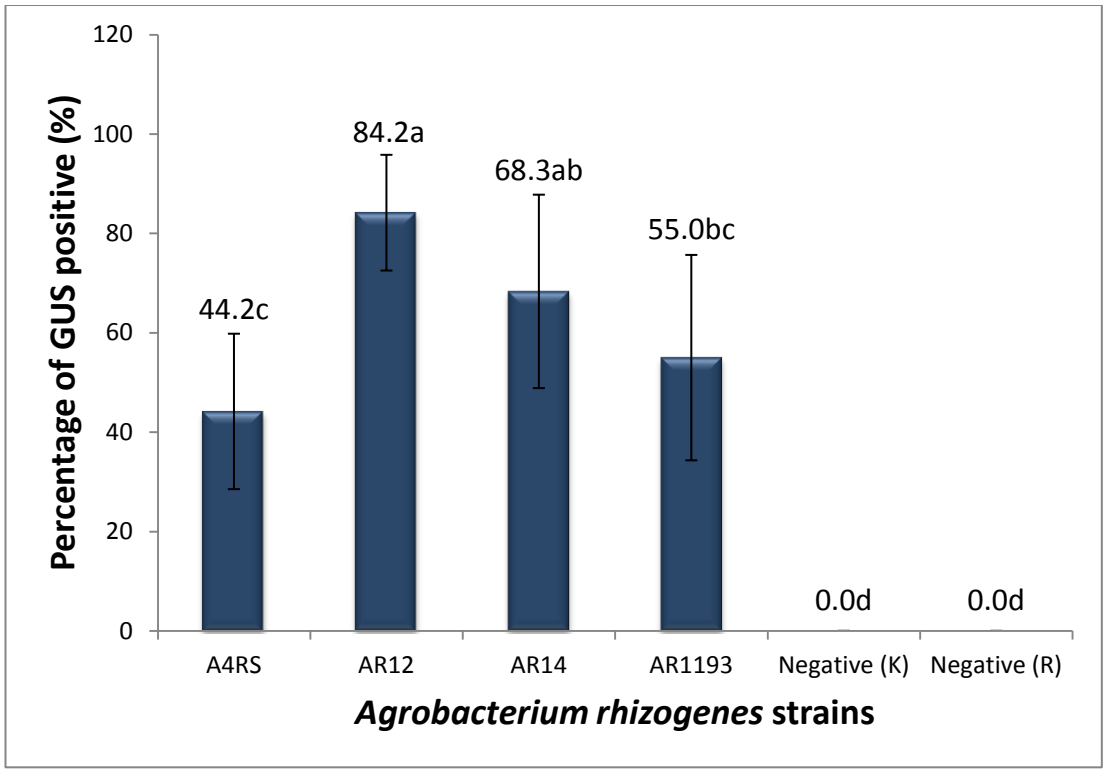

Figure 4.3: **Frequency of transient GUS expression on *Eurycoma longifolia* somatic embryos transformed with *Agrobacterium rhizogenes* strains A4RS, AR12, AR14, AR1193 and negative controls with kanamycin (K) and rifampicin (R).** Error bars show standard deviation. Mean with different alphabet on top is significantly different (Games-Howell's Test, $\alpha = 0.05$).

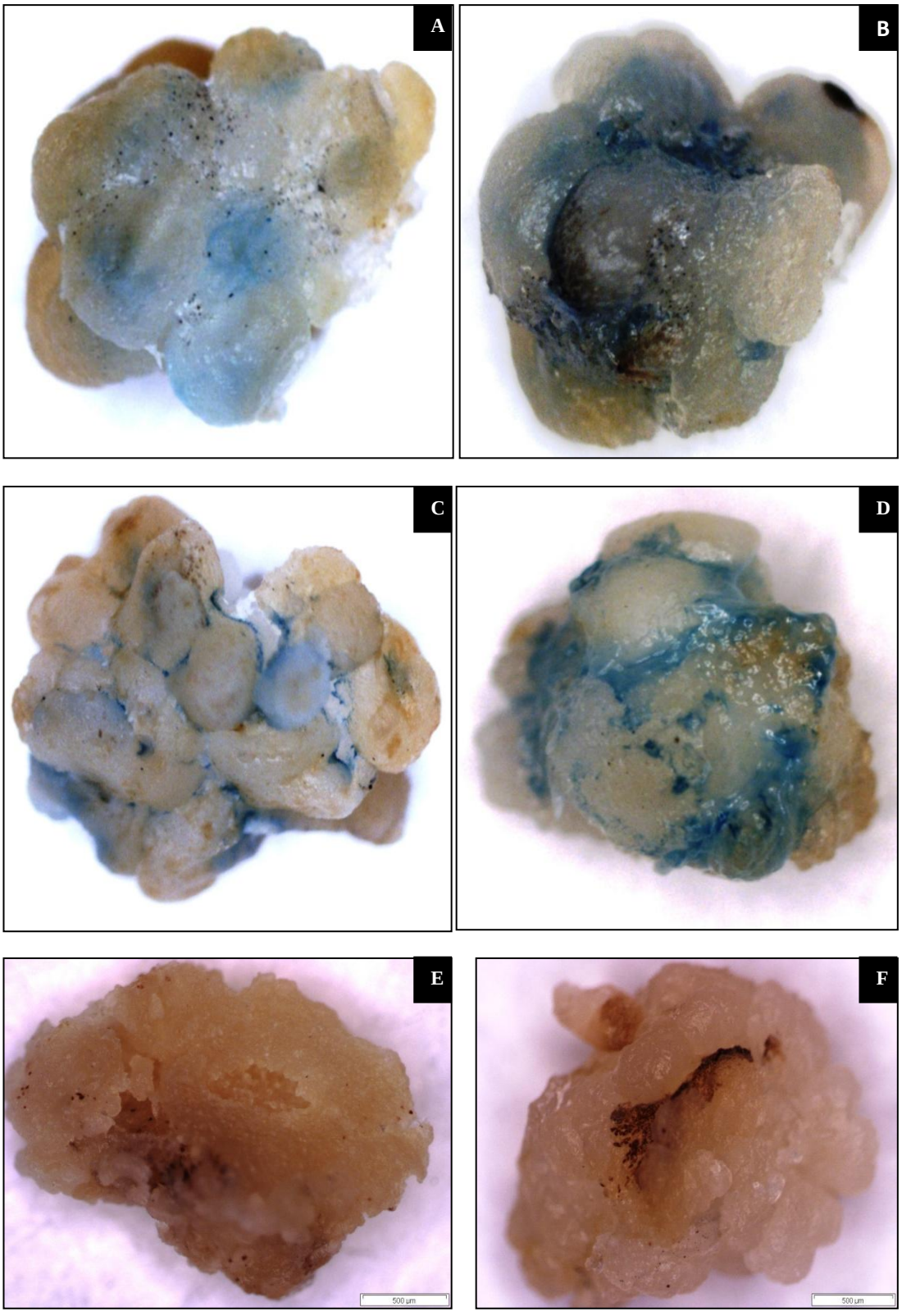

Plate 4.13: Transient GUS expression of *Eurycoma longifolia* somatic embryos transformed with *Agrobacterium rhizogenes* strains. (A) A4RS **(B)** AR12 **(C)** AR14 **(D)** AR1193 **(E)** Negative control (kanamycin) and **(F)** Negative control (rifampicin).

4.5.2 Indole-butyric acid (IBA) concentrations

As shown by Fig. 4.4, the highest GUS positive frequency was observed for 1.5 mg/L IBA at 84.2% followed by 1.0, 2.0, 0.0 and 0.5 mg/L IBA. There are no significant differences between the explants co-cultivated in mediums containing 1.0, 1.5 and 2.0 mg/L IBA. There are also no significant differences in GUS positive percentages for 0.0, 1.0 and 2.0 mg/L IBA. Explants co-cultivated in mediums containing 0.0, 0.5 and 2.0 mg/L IBA were not significantly different as well. Since there are no significant differences in GUS positive results shown for 1.0, 1.5 and 2.0 mg/L IBA, 1.0 mg/L IBA were chosen for the subsequent optimisation step due to the lower concentration required for the medium formulation and to avoid any formation of false positive 'hairy root' caused by higher concentrations of the phytohormone. Plate 4.14 shows the transient GUS expression of *Eurycoma longifolia* somatic embryos transformed with *Agrobacterium rhizogenes* strain AR12 on co-cultivation medium containing various concentrations of IBA. Plate 4.15 shows *Eurycoma longifolia* somatic embryo transformed with *Agrobacterium rhizogenes* strain AR12 on co-cultivation medium containing 1.0 mg/L IBA. A thin strand of tissue resembling a root structure and stained blue is seen protruding from the explant.

4.5.3 Dithiothreitol (DTT) concentrations

Fig. 4.5 shows that the highest GUS positive percentage was achieved for 4.0 mg/L DTT followed by 3.0, 0.0, 1.0 and 2.0 mg/L of DTT. There are no significant

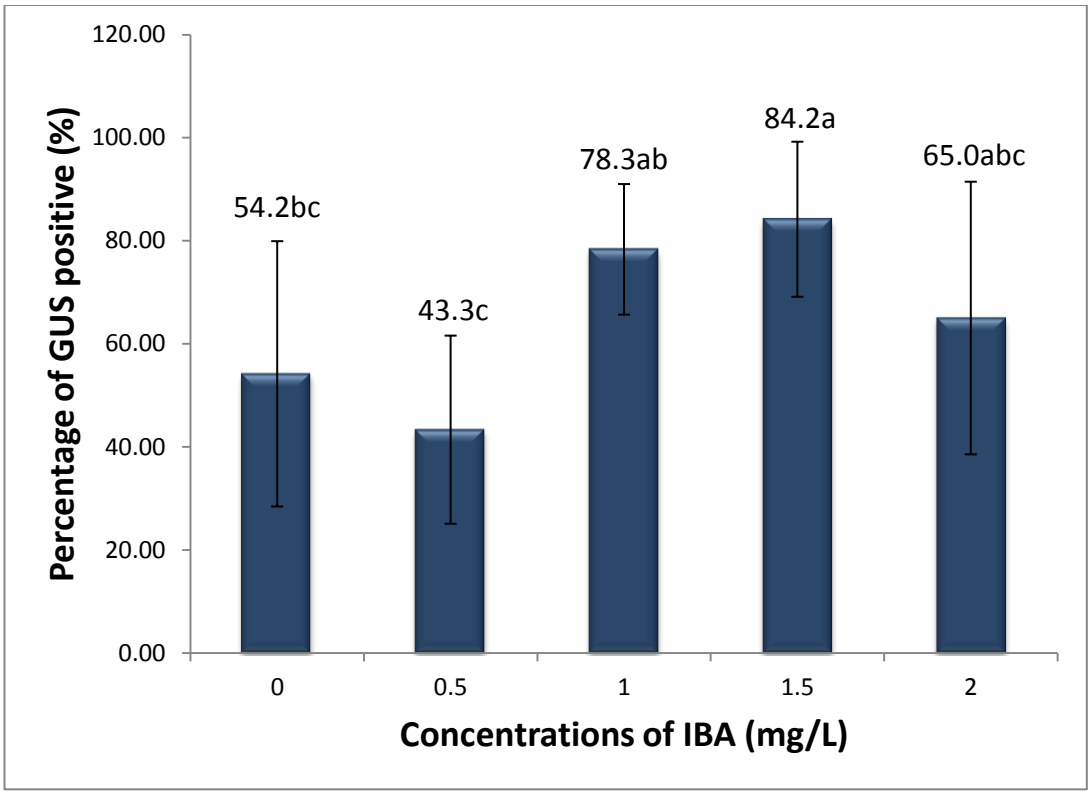

Figure 4.4: **Frequency of transient GUS expression on *Eurycoma longifolia* somatic embryos transformed with *Agrobacterium rhizogenes* strain AR12 on different concentrations of the hormone IBA.** Error bars show standard deviation. Mean with different alphabet on top is significantly different (Games-Howell's Test, $\alpha = 0.05$).

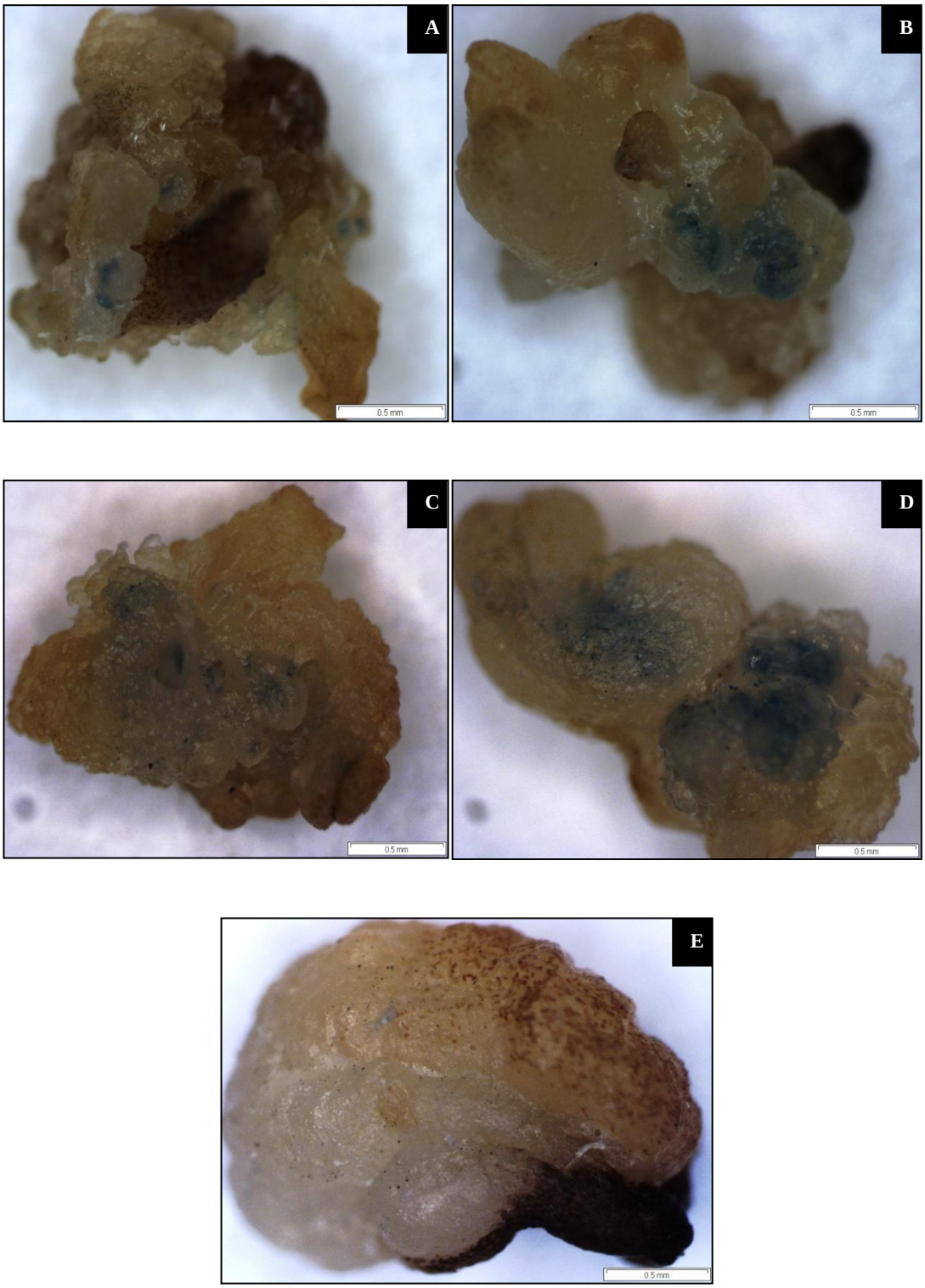

Plate 4.14: Transient GUS expression of *Eurycoma longifolia* somatic embryos transformed with *Agrobacterium rhizogenes* strain AR12 on co-cultivation medium containing IBA. (A) 0.0 mg/L **(B)** 0.5 mg/L **(C)** 1.0 mg/L **(D)** 1.5 mg/L **(E)** 2.0 mg/L.

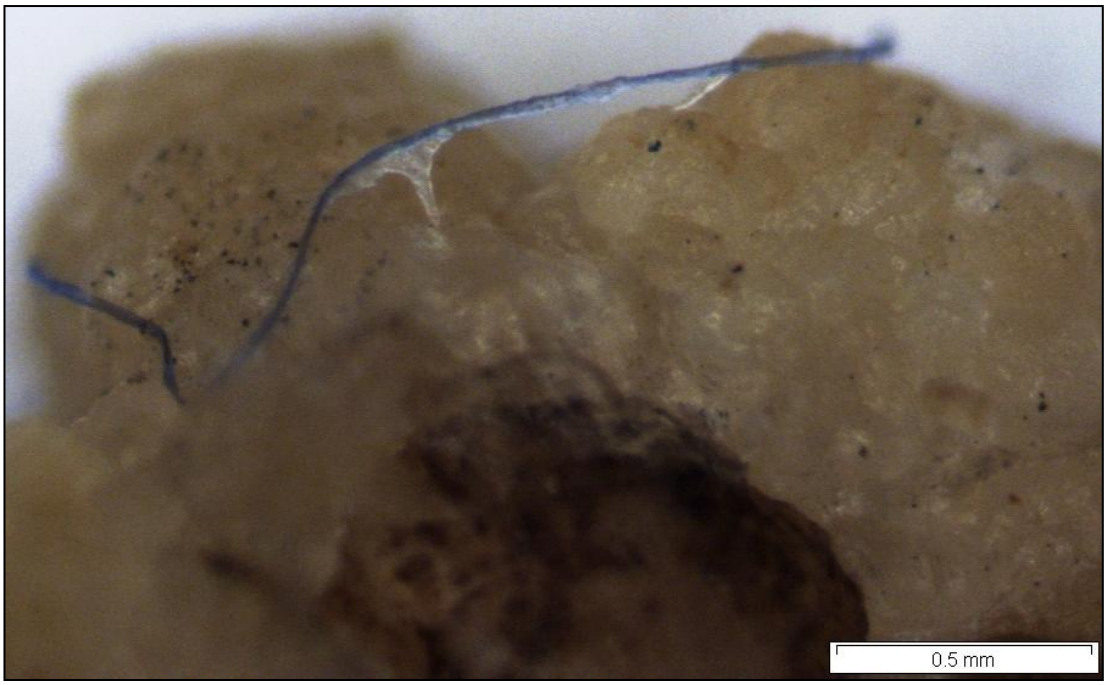

Plate 4.15: ***Eurycoma longifolia*** **somatic embryo transformed with** ***Agrobacterium rhizogenes*** **strain AR12 on co-cultivation medium containing 1.0 mg/L IBA.** A thin strand of tissue resembling a root structure and stained blue is seen protruding from the explant.

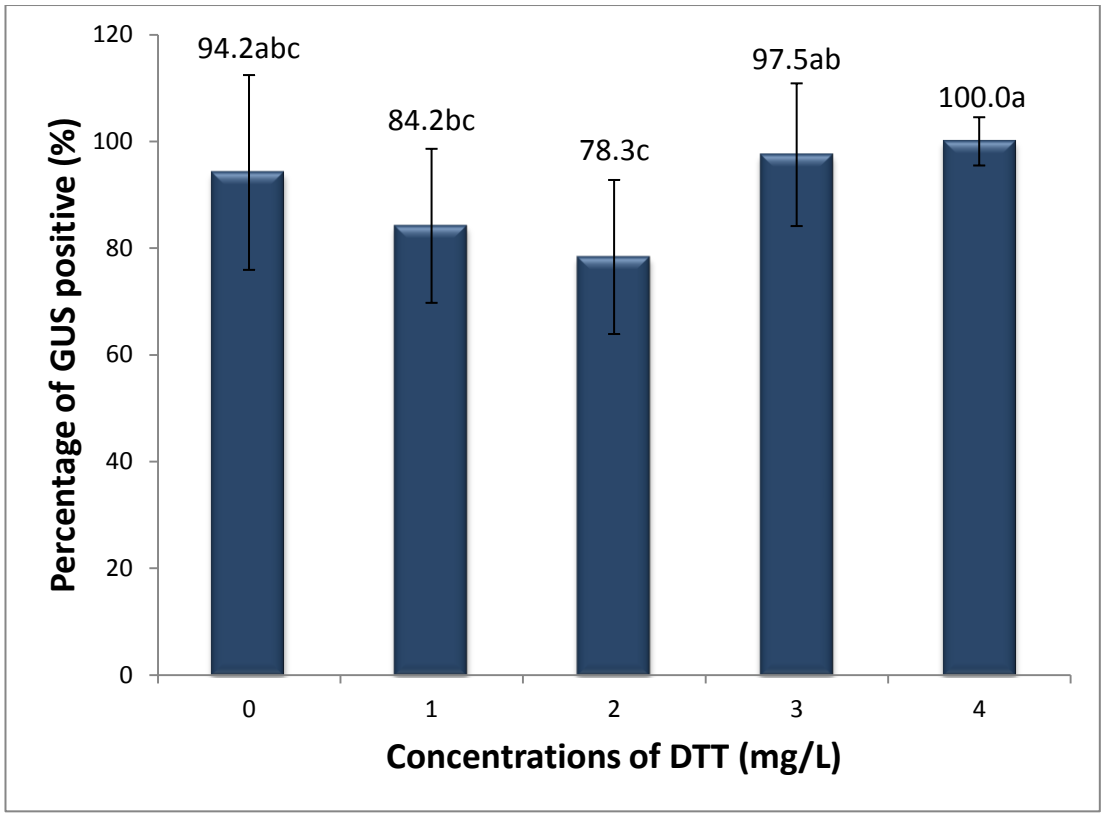

Figure 4.5: **Frequency of transient GUS expression on *Eurycoma longifolia* somatic embryos transformed with *Agrobacterium rhizogenes* strain AR12 on different concentrations of the antioxidant DTT.** Error bars show standard deviation. Mean with different alphabet on top is significantly different (Games-Howell's Test, $\alpha = 0.05$).

differences between 0.0, 3.0 and 4.0 mg/L DTT. There are also no significant differences observed for 0.0, 1.0 and 3.0 mg/L DTT. There are no significant differences observed between 0.0, 1.0 and 2.0 mg/L DTT. The GUS positive percentage shows a reduction from 0.0 to 1.0 and 2.0 mg/L DTT. The GUS positive percentage then rises dramatically from 2.0 to 3.0 mg/L before achieving 100% GUS positive percentage at 4.0 mg/L DTT as shown in Figure 4.5. These changes were determined to detrimental to the plant and due to there being no significant difference between GUS positive percentages in the medium devoid of DTT and those with the highest concentrations, DTT was removed from the subsequent optimisation step. Plate 4.16 shows the transient GUS expression of *Eurycoma longifolia* somatic embryo transformed with *Agrobacterium rhizogenes* strain AR12 on co-cultivation medium containing various concentrations of DTT.

4.5.4 Polyvinylpyrrolidone (PVP) concentrations

Fig. 4.6 shows that the highest GUS positive percentages were achieved for 1% (w/v) PVP followed by 2, 0, 3 and 4% (w/v) PVP. There are no significant differences between GUS positive results observed for 0, 1 and 2% (w/v) PVP. Likewise, there are no significant differences observed for 0, 2, 3, and 4% (w/v) PVP. PVP at 1% (w/v) yielded the highest GUS positive percentage at 94.2% and was chosen as the optimised PVP concentration for the co-cultivation medium formulation. Plate 4.17 shows the transient GUS expression of *Eurycoma longifolia* somatic embryo transformed with *Agrobacterium rhizogenes* strain AR12 on co-cultivation medium containing various concentrations of PVP.

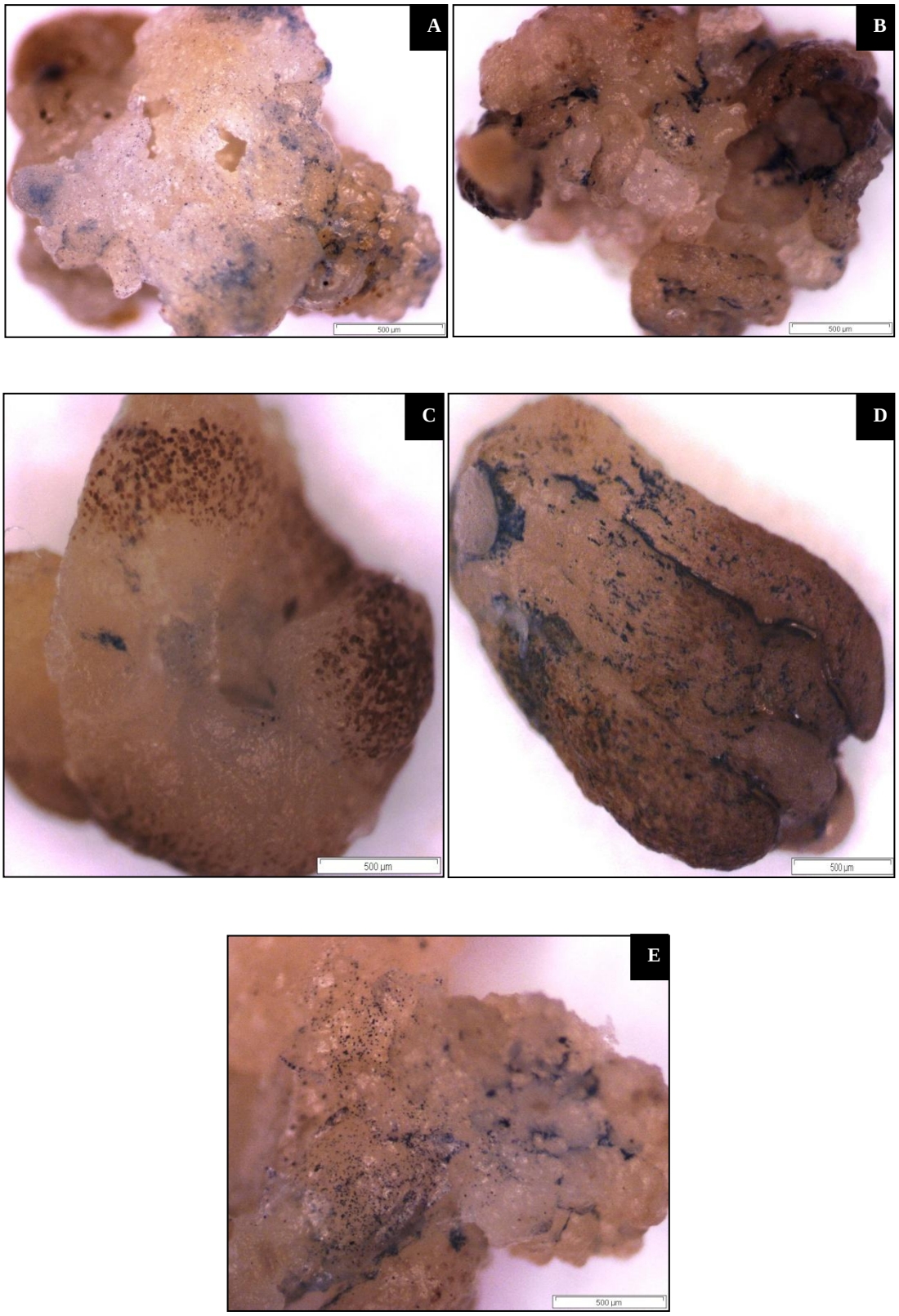

Plate 4.16: Transient GUS expression of *Eurycoma longifolia* somatic embryo transformed with *Agrobacterium rhizogenes* strain AR12 on co-cultivation medium containing DTT. (A) 0.0 mg/L **(B)** 1.0 mg/L **(C)** 2.0 mg/L **(D)** 3.0 mg/L **(E)** 4.0 mg/L.

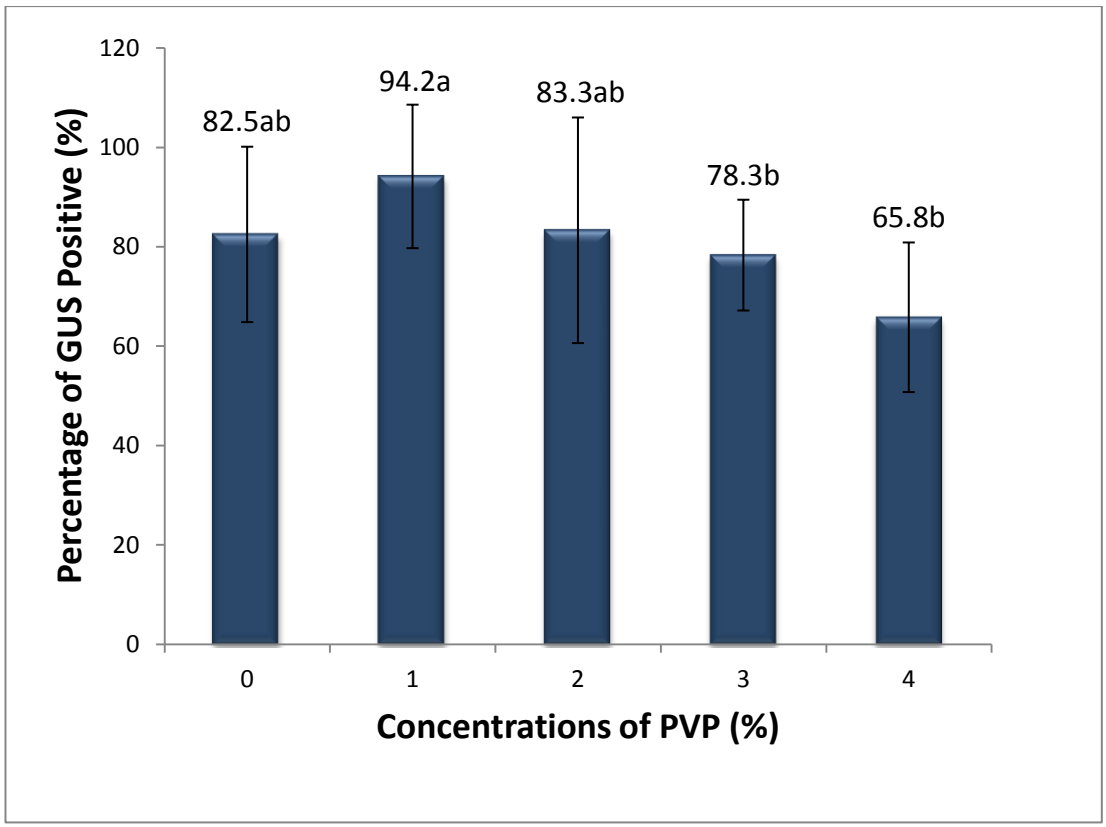

Figure 4.6: **Frequency of transient GUS expression on *Eurycoma longifolia* somatic embryos transformed with *Agrobacterium rhizogenes* strain AR12 on different concentrations of the antioxidant PVP.** Error bars show standard deviation. Mean with different alphabet on top is significantly different (Games-Howell's Test, $\alpha = 0.05$).

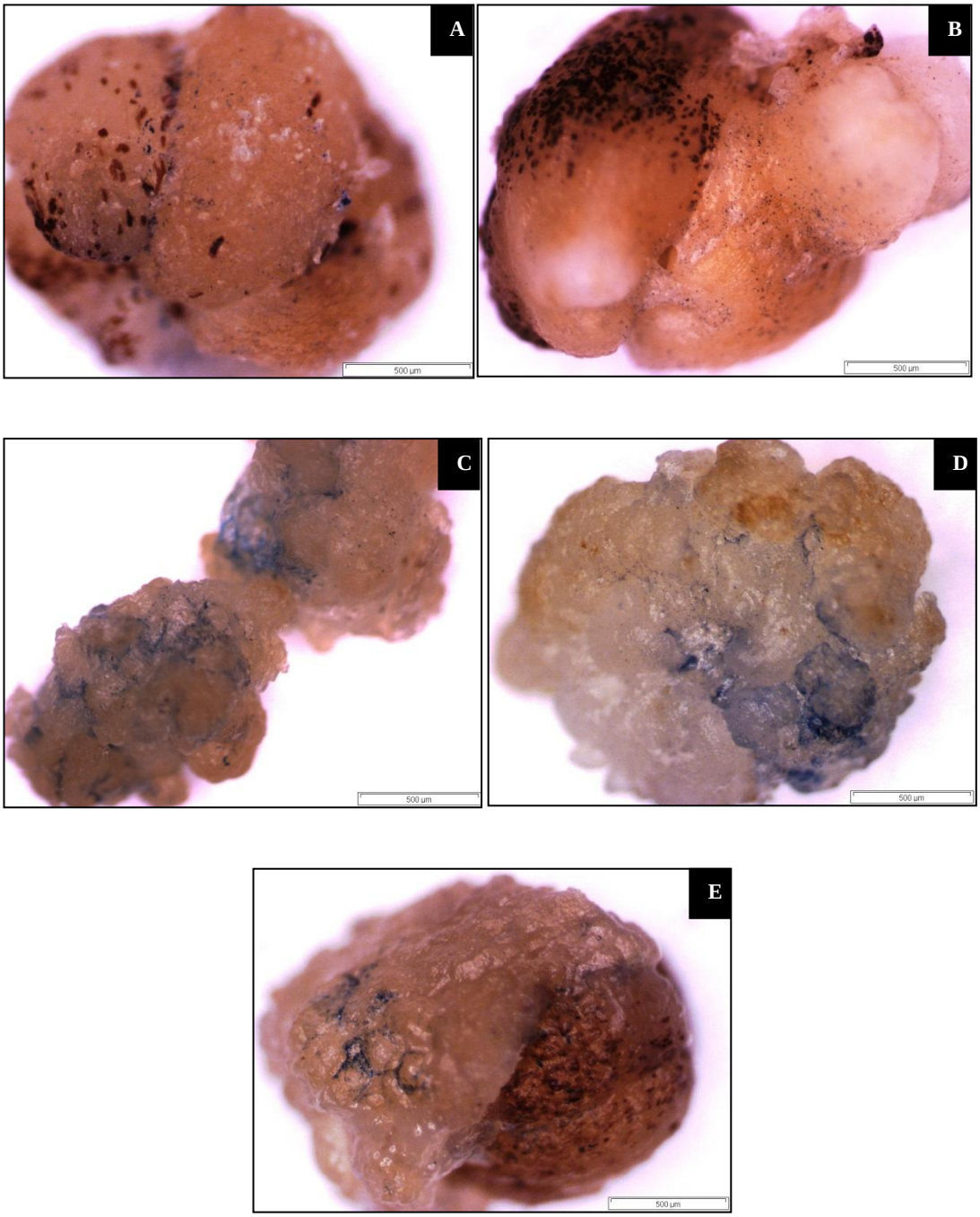

Plate 4.17: Transient GUS expression of *Eurycoma longifolia* somatic embryos transformed with *Agrobacterium rhizogenes* strain AR12 on co-cultivation medium containing PVP. (A) 0% **(B)** 1% **(C)** 2% **(D)** 3% and **(E)** 4%.

4.6 Hairy root induction in somatic embryos of *Eurycoma longifolia*

Based on the results obtained from the optimisation step, the strain chosen for *Agrobacterium*-mediated transformation of *Eurycoma longifolia* somatic embryos is AR12. The medium formulation for both pre-culture and co-cultivation steps is as follows: full strength MS medium were fortified with 1.0 mg/L IBA, 1% (w/v) PVP, 3% (w/v) sucrose and solidified with 2.8 g/L Gelrite^{TM}. This medium formulation was then used for the induction of hairy roots from somatic embryo explants of *Euryoma longifolia*. Four to five (4-5) week old somatic embryo explants (4-6 mm) were selected and pre-cultured for two (2) days in the medium stated above. Pre-cultured explants were injured by transverse cutting and transformed with *A. rhizogenes* strains AR12 by immersion for 20 minutes, blotted and transferred back into the same medium as above. Explants were co-cultivated for 3 days in the dark at 24 ± 0.2°C. Co-cultivated explants were washed with 500 mg/L cefotaxime sodium and transferred into the same medium for four (4) days in the dark at 24 ± 0.2°C, following which the explants were observed for *Agrobacterium* overgrowth on the surface of the explants. The explants were washed with 500 mg/L cefotaxime in the event of overgrowth and transferred back into a fresh medium as formulated above.

However, several observations regarding the induction process were made at this juncture. Firstly, prolonged culture of the embryo explants in the dark is detrimental to the plant as the process of photosynthesis is disrupted. The explants were turning yellow from their natural green state. Therefore, the explants were transferred to tissue culture room conditions of 16 hours photoperiod in 25 ± 2°C.

Additionally, there was consistent overgrowth of *A. rhizogenes* on the surface of the embryo explants despite constant washing with cefotaxime.

Alternative methods of controlling the bacterial overgrowth were investigated and the addition of activated charcoal was ascertained to be a viable choice for controlling the overgrowth and promoting root induction from transformed explants. Activated charcoal (1.0 g/L) was also added to the medium while all the other medium components were maintained. Following this step, a reduction in the overgrowth of *A. rhizogenes* cultures were observed. The plants remained green indicating that the activated charcoal was not detrimental to the explants. The period between the washing steps were prolonged, reducing the frequency with which the explants are exposed to the high concentration of cefotaxime. Nevertheless, overgrowth of *A. rhizogenes* was still being observed.

To enhance the efficacy of the antibiotic during the washing step, 5.0 mg/L silver nitrate was added to the liquid medium. It was noted that this was a step in the right direction as the overgrowth was eliminated completely by the addition of both activated charcoal in the semi solid culture medium and silver nitrate during the antibiotic washing step. The explants remained green throughout this process despite there being no induction of hairy roots from the transformed explants.

4.7 Detection of transformants using polymerase chain reaction (PCR)

Genomic DNA was extracted from the transformed and control somatic embryos using the Genomic DNA Mini Kit (Plant; Geneaid Biotech Ltd., Taipei

County, Taiwan, Republic of China) and the DNA purity and concentration was determined. Molecular analysis using PCR was conducted for the detection of *rol* and *vir* genes in the transformed plant.

Plate 4.18 shows the gel electrophoresis image for the detection *rolA, rolB, rolC* and *virD* genes in the transformed somatic embryos. The lane marked M was reserved for the 100bp DNA marker. Lanes 1, 4, 7 and 10 were used for the negative control of PCR reaction mixture with water for *rolA, rolB, rolC* and *virD* primers, respectively. Lanes 2, 5, 8 and 11 contain the genomic DNA sample for the detection of *rolA, rolB, rolC* and *virD* from untransformed, control somatic embryos. Lanes 3, 6, 9 and 12 were used for the detection of the *rol* and *vir* genes from the transformed somatic embryos.

Plate 4.18 shows the successful integration of the *rol* and *vir* genes from *Agrobacterium rhizogenes* strain AR12 into somatic embryos of *Eurycoma longifolia* as seen in lanes 3, 6, 9 and 12. Bands were not observed in both the PCR reaction mixture control as well as the lanes containing genomic DNA template from untransformed somatic embryos indicating that the presence of the gene is a direct result of the transformation protocol. Lane 3 which shows a faint *rolA* band between the 200 and 300 bp marker bands is an indication that the 248 bp *rolA* gene has been amplified although the expression is not very intense. The amplification of *rolB* could be clearly observed in lane 6, between the 700 to 800 bp marker band, corresponding to the expected size of the 780 bp gene. The 490 bp *rolC* band could be observed in lane 9, albeit not very clearly as that of the 450 bp *virD* gene in lane 12.

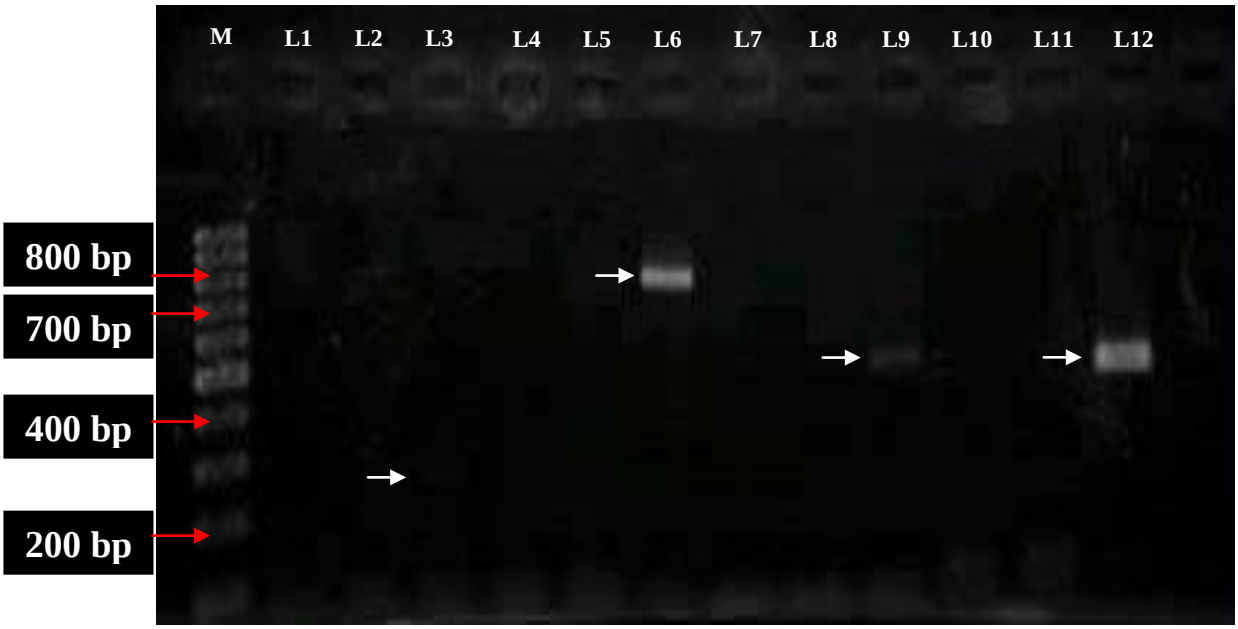

Plate 4.18: Molecular analysis of *rolA*, *rolB*, *rolC* and *virD* genes integration into *Eurycoma longifolia* somatic embryos. M = Marker; **L1** = Master mix control for *rolA* with water; **L2** = Non-transformed somatic embryo control for *rolA*; **L3** = Transformed with *rolA* (248bp); **L4** = Master mix control for *rolB* with water; **L5** = Non-transformed somatic embryo control for *rolB*; **L6** = Transformed with *rolB* (780bp); **L7** = Master mix control for *rolC* with water; **L8** = Non-transformed somatic embryo control for *rolC*; **L9** = Transformed with *rolC* (490bp); **L10** = Master mix control for *virD* with water; **L11** = Non-transformed somatic embryo control for *virD*; **L12** = Transformed with *virD* (450bp).

CHAPTER 5

DISCUSSION

5.1 Micropropagation of *Eurycoma longifolia*

5.1.1 Callus induction from *in vivo* and *in vitro* leaf explants

The primary purpose of this study is to develop callus cultures and multiply them *in vitro* to be used for subsequent transformation experiment. One of the problems faced during the callus induction process was the recurrent appearance of fungal contamination from the surface sterilised explants, even after a prolonged period of culture. However, during the collection of leaf explants from the *in vivo* plant, it was noted that the leaves did not display any outward signs of a pathological response to a fungal infection. The leaves appeared green and healthy. The contamination may be attributed to the presence of intracellular fungal hyphae within the leaf tissue that emerge in the culture following the intensive surface sterilisation protocol.

A variety of microorganisms form mutualistic and parasitic endosymbioses relationships with plants. These infiltrations are defined by microbial symbionts that live partly or exclusively within living plant cells following the infiltration process (Parniske, 2000). Mutualistic symbiosis of plant roots by arbuscular mycorrhiza (AM), mildew and rust diseases are some of the most extensive endosymbiotic interplay between plants and fungal symbionts (Bonfante and Genre, 2010). The primary feature of endosymbiosis is the presence of a host membrane forming a

chamber known as ‘symbiosome’ which encloses the symbiotic microorganism within host cells (Parniske, 2000). In such instances, the fungal symbiont would be protected from the effects of surface sterilisation by the plant membrane. The establishment in plant tissue culture systems may result in the immediate expression of the contaminants or could remain dormant until more favourable conditions are present before manifesting on the plant and growth media (Leifert and Cassells, 2001).

Callus induced from the *in vivo* explants did not emerge until approximately 3 months from the culture period whereas those induced from *in vitro* explants took a short 3 weeks before the first callus beads were observed on the surface of the leaves. This may be due to the time needed by the *in vivo* explants to acclimatise to *in vitro* conditions before inducing callus as a response to injury. The quality of the callus was not up to par with those from *in vitro* leaves and more significantly, failed to grow in the medium following sub-culture. Contrarily, callus from *in vitro* leaves were light yellow in colour, was friable and was able to grow in the medium. However, these explants were slow growing and were not used for the transformation experiment as there was a severe lack of explants.

5.1.2 Induction and multiplication of somatic embryos

The induction and multiplication of somatic embryos were achieved on full strength MS medium containing 1.0 mg/L 2,4-D and 0.5 mg/L kinetin, with the addition of 1.0 g/L activated charcoal for the multiplication medium (Sobri *et al.*, 2005a). The medium was proven to be excellent in supporting the growth of the

somatic embryos for up to 10 weeks at a time. Periodic sub-cultures in the same medium allowed the multiplication of somatic embryos sufficient for the subsequent experiments. Culture periods longer than 10 weeks usually resulted in the embryos reverting back into embryogenic callus stage, possibly due to the stress induced by the limited nutrient and space available in the culture vessel at this point. Injuring the embryos during the sub-culturing resulted in the formation of more somatic embryos suggesting that injury plays an important role in the induction of somatic embryogenesis. It has also been reported that the addition of 2,4-D in culture medium could induce somatic embryogenesis (Karami and Saidi, 2010). The supplementation of auxin 2,4-D with cytokinin kinetin is required for optimum response of the plant and to prevent necrosis of embryogenic callus (Sharma, 2009).

5.1.3 Propagation of *in vitro* shoot cultures

In vitro shoot cultures were multiplied from pre-existing cultures supplied by FAS using full strength MS medium containing 5.0 mg/L kinetin (Sobri *et al.*, 2005b). The leaves and stem from these cultures were used for the subsequent transformation protocol.

5.1.4 Surface sterilisation of seeds and growth of *in vitro* plantlets

Freshly collected fruits of *Eurycoma longifolia* were surfaced sterilised and germinated in basal full strength MS semi solid medium without the addition of phytohormones (Murashige and Skoog, 1962). Extensive fungal contamination was observed in the surface sterilised explants, prompting many to be discarded. Others, while free from contamination, simply failed to germinate and this may be due to the immature state of the zygotic embryo (Sobri *et al.*, 2005b). Root initials were seen emerging from the cotyledons as early as 2 weeks from incubation in the medium followed by the emergence of shoots. The cultures were transferred into full strength MS medium containing 5.0 mg/L kinetin to induce the seedlings into forming shoots. The leaf, stem and root from these cultures were used for the subsequent transformation protocol.

5.2 Microscopy observations of somatic embryos

5.2.1 Histological observations

Somatic embryos were propagated in full strength MS medium supplemented with 2,4-D, kinetin and activated charcoal (Sobri *et al.*, 2005a). The somatic embryos of *Eurycoma longifolia* were confirmed using histological analysis. Each non-zygotic embryo or embryoid is a bipolar structure, consisting of plumular and radicular ends, thus they closely resemble their zygotic counterpart. Usually, the same pattern of growth is observed in cultured somatic embryos, passing through the three sequential

stages of embryo formation, such as globular stage, heart-shaped stage and torpedo stage. It is the torpedo stage which is a bipolar structure and gives rise to a complete plant (Sharma, 2009). A common mistake in literature regarding embryogenesis is the failure to demonstrate a well organised shoot meristem. Simply showing a structure with the shape of an embryo is not good enough. The lack of a suitably organised shoot meristem is often the reason why many somatic embryos could not grow into a complete plant. It is imperative that through proper sectioning and staining, well organised apical meristems should be indeed present. This is a good indication of high quality somatic embryos (Yeung, 1999).

Cytohistologic structure, size and shape vary in vegetative shoot apices. Shoot apex of dicotyledonous angiosperms such as *E. longifolia* possess tunica-corpus organization with up to five layers of tunica being observed and recorded (the narrow layer of cells that borders shoot apical meristem in Plate 4.5) (Pandey, 2007). The order of root meristematic cells usually follows a distinctive design specific to the species during the development of the embryo (Plate 4.6). Their distinguishing features include compact, protoplasmic cells that experience rapid cell division. The tissues of the mature root are eventually derived from a number of these cells of the apical meristem, which are termed as root initials (Pandey, 2007).

Somatic embryos of *Eurycoma longifolia* have been shown to resemble the developmental pattern of seeds or zygotic embryos found in the wild as they possess well organized shoot and root apical meristems. Cultured apical meristems of *E. longifolia* are suitable for transformation purposes because these can be easily regenerated into whole plants. Intact DNA taken up by the plant appears to be rapidly transported to meristematic regions where growth and differentiation are centred (Pandey, 2007). This allows the successful assimilation and execution of T-DNA

activity in transformed organisms. Histological studies of somatic embryos of *E. longifolia* suggest that genetic transformation with *Agrobacterium rhizogenes* is highly possible due to the presence of actively growing apical meristems which are similar to the meristematic regions seen in developing zygotic embryos.

5.2.2 SEM observations

The surfaces of somatic embryo explants were viewed using ESEM technique in two different variations. In the first viewing, the fresh explants were obtained from the multiplication medium and viewed in their native state without any prior treatment. In the second, the explants were fixed with 1% osmium tetroxide for an hour before viewing with ESEM. Observations of the somatic embryos show that the explants were quickly dehydrated by this technique of viewing. The cells on the surface appeared to collapse inwardly due to the rapid loss of moisture in the high pressure chamber within the microscope (Plate 4.8). However, embryos that were fixed prior to viewing were able to hold their structural integrity for a longer period of time compared to those without any prior fixing (Plate 4.9).

Hydrated, insulating samples could be viewed imaged under an electron beam using the environmental scanning electron microscopy (ESEM) technique (Danilatos, 1993). The clarity of images taken with this technique is lesser than conventional scanning electron microscopy (CSEM) but superior to those of optical microscopy. Minimal sample preparation is needed for the technique, a considerable advantage that allows a quick observation and the images are less prone to exposure of foreign elements that may be introduced by the extensive sample preparation usually required for CSEM. Precise control of both the beam energy and humidity in the

microscope chamber are nevertheless essential to prevent dehydration resulting in damaged samples. Viewing live cells are made possible using ESEM, as shown by the present study (McGregor *et al.*, 2013).

5.3 Interaction between *Agrobacterium rhizogenes* and *Eurycoma longifolia*

5.3.1 Chemotaxis movement assay

The results of the chemotaxis assay show that there was positive chemotactic response towards the embryo explant by all the strains tested, irrespective of wounding level (Figures 4.1 and 4.2). Sloppy agar plates made using a reduced quantity of agar are known as swarm plates. The concentration gradient is created when the *Agrobacterium* is inoculated at the centre of such plates due to the continuous utilisation of nutrient by the growing bacteria. The bacteria respond to this gradient by steadily moving outward, consuming nutrients and creating more gradient as they go. Swarm plates have been shown to possess many applications (Shaw, 1995). The positive chemotactic response shows that the plant and its exudates serve as an attraction for the movement for *A. rhizogenes* strains in the swarm plate medium.

Capillary assay method could also be used to study chemotaxis movements of *Agrobacterium* (Perez Hernandez *et al.*, 1999). Even though it is said to be precise, the ability to record reliable results are made difficult by the complicated assembly and the ample number of replicates needed for the assay. The collection of *Agrobacterium* cells in the culture due to the production of extracellular polysaccharides complicates the process of accurately quantifying the bacteria in the

sample (Subramaniam *et al.*, 2009). Thus, the chemotaxis assay was conducted using the swarm plate method.

5.3.2 SEM observation of bacterial attachment

Somatic embryos co-cultivated with *Agrobacterium rhizogenes* strains were fixed with 1% osmium tetroxide, freeze-dried, coated with gold particles, mounted on a sample stub and viewed using CSEM. Observations are shown in a sequential manner in Plate 4.10. In the first image (Plate 4.10A and B), the bacteria were seen forming a layer of cells on the surface of the explants in a web-like structure. On closer inspection (Plate 4.10C), the rod shaped *A. rhizogenes* cells were seen to be attached by fibrillar connections to each other and are closely packed together. Attachment of the bacterial cells could be seen in Plate 4.10D. Strands could be seen running to the plant cell wall and there were several strands attached to the bacterial cell.

The bacterial distribution on the surface of the explants was not uniform. It was postulated in a previous study that developmental stage of the explants may influence the binding ability of the *Agrobacterium* cell (Graves *et al.*, 1988). This would reduce the transformation efficiency significantly, particularly if T-DNA transfer to individual cells is contingent on the attachment of a population of bacteria.

5.4 *Agrobacterium rhizogenes*-mediated transformation of *Eurycoma longifolia*

5.4.1 Hairy roots induction from *in vivo* leaves, rachis and *in vitro* leaves, stems and roots of *Eurycoma longifolia*

Various explants of *Eurycoma longifolia* such as *in vivo* leaves, rachis and *in vitro* leaves and roots were investigated for the initiation of hairy roots but roots failed to emerge from these explants. Surface sterilised explants underwent browning and necrosis following the protocol indicating that the explants were affected negatively and quite possibly had lost their growth potential. *In vitro* explants turned yellow after the transformation was carried out. This is an indication that the plant's defence mechanisms have been initiated to fend off the plant pathogenic *A. rhizogenes* cells.

Three weeks after the transformation was carried out, observations showed the development of callus tissues at the bottom of the stem explants transformed with *Agrobacterium rhizogenes* strain AR14. Soon thereafter, the appearance of root initials was noted. Hairy root-like structures emerged from the base of the stem explants and continued to grow upward, indicating that it is non-geotropic in nature. Of the four (4) tested *Agrobacterium rhizogenes* strains, only AR14 was able to generate roots from the stem explants.

5.5 Optimisation of transformation efficiency in *Eurycoma longifolia* somatic embryos

The improvement of a given crop species using plant genetic transformation makes the development of an efficient method a necessity. Various factors that influence *Agrobacterium*-mediated transformation of plants have been examined and explained. Plant species, genotype, bacterial strains, cell density, and the addition of growth regulators, antibiotics and acetosyringone are some of the factors that have been investigated (Karami, 2008). The critical point in developing an efficient transformation protocol is to find the optimal conditions of the many factors that act together during transformation.

5.5.1 Virulence of *Agrobacterium rhizogenes* strains

Of the various factors influencing transformation efficiency, one of the most important is the infecting ability of the *Agrobacterium* strain. In this optimisation, four *Agrobacterium rhizogenes* strains were utilised in the transformation of *Eurycoma longifolia* somatic embryos. The resistance to rifampicin and spectinomycin antibiotics were incorporated into the A4 wild strain and renamed as A4RS (Jouanin *et al.*, 1986). Electroporation method was used to introduce the binary vector pBIN19 into A4RS (Sambrook *et al.*, 1989). The β-glucuronidase gene derived from *Escherichia coli* is incorporated into the T-DNA of the pBIN19 plasmid with an additional intron for specific expression in plants (Vancanneyt *et al.*,

1990) and the cauliflower mosaic virus (CaMV) 35S promoter and terminator regulates its expression. GUS assay was used to determine the translocation efficiency of the plasmid in the armed A4RS-p35S-*gusA-int* strain (Alpizar *et al.*, 2006). Three (3) *A. rhizogenes* strains AR12, AR14 and AR1193 were modified to carry the pBR322 plasmid with the *gusA* reporter gene while AR14 is fitted with an additional chloramphenicol acetyltransferase (CAT) reporter gene. The pBR322 derived cloning vector, pIV10 could be utilised for the conjugational translocation and assimilation of gene constructs in these strains. The pIV10 plasmid is 4.5 kb and carries the *Eco*RI-*Hind*III polylinker sites from pUC19 (Radutoiu *et al.*, 2005). For ease of purpose, transient GUS expression was used as the standard assay to determine the transformation efficiency. All four strains used for the transformation experiments are agropine type strains, known for their hyper-virulent and non polar nature of infection (Veena and Taylor, 2007).

The different strains of *Agrobacterium rhizogenes* tested show a markedly different transient GUS expression frequency as shown in Figure 4.3. The transformation efficiency of AR12 is the highest with 84.2% followed by AR14 with a transformation frequency of 68.3%. There are no significant differences between these two strains. A4RS had the lowest transformation frequency (44.2%) but did not have any significant difference with the AR1193 strain (55%). This is an interesting finding as previous reports show that A4RS is a highly virulent strain and has been successfully used for the initiation of hairy roots in coffee plants, *Coffea arabica*, in which it achieved 80% transformation frequency (Alpizar *et al.*, 2006). *Agrobacterium rhizogenes* strains AR12 and AR1193 have previously been used for the transformation of *Lotus japonicus* and *Medicago trunculata* and the resulting root

cultures were used to study Nod-factor receptor genes in the root nodules (Radutoiu *et al.*, 2007).

Analysing the embryo explants displaying transient GUS expression for all the four strains show no marked difference between the intensity of the blue staining. Negative control explants did not display any blue staining in all the explants used, indicating that the blue staining seen in the putative transformants are indeed the product of *gusA* gene activity successfully translocated into the host genome by the *A. rhizogenes* strains. The 35S CaMV plant promoter is directing the synthesis of β-glucuronidase enzyme needed to break down the X-Gluc compound introduced during the GUS assay (Jefferson, 1987). In a study conducted on four *Hyoscyamus* species, the virulence as well as the morphology of roots induced by five different strains of *A. rhizogenes* showed variability in their root-inducing capacity (Akramian *et al.*, 2008).

The transformation ability of diverse strains of *A. rhizogenes* varies as dictated by strain specificity observed in the present study. This confirms the theory that infection capabilities of different *A. rhizogenes* strains would differ from one another. The plasmids within the bacterial strains and the *vir* genes that they encode could help to explain the difference in virulence. Using trans-conjugational methods of plasmid transfer, it was shown that virulence of *Agrobacterium rhizogenes* strain 15834 is encoded by the plasmid carried within the bacterial cell (White and Nester, 1980). It was noted that *A. rhizogenes* strain 15834 is the parent strain for disarmed strains AR12, AR14 and AR1193. Subsequently, *A. rhizogenes* strain AR12 was chosen for the transformation optimisation experiments based on these observations due to the high frequency of the transient GUS expression recorded.

5.5.2 Indole-butyric acid (IBA) concentrations

Out of the four identified types of Ri plasmid, only those belonging to the agropine group encodes for auxin (*aux*) genes in its T_R-region. Initiation of hairy roots in transformed cells could be explained by T-DNA controlled auxin synthesis. However, the T-DNA harboured by the other types of Ri plasmid are highly similar to the T_L region of agropine Ri plasmids proving that hairy roots can be initiated by T-DNA without the presence of *aux* genes (Cardarelli *et al.*, 1987). However, agropine strains differ from the other groups in their ability to induce roots without any preference for the polarity of the discs, and is therefore classified as non polar (Veena and Taylor, 2007). The development of hairy roots by polar *A. rhizogenes* strains in explants has been attributed to the basipetal transport of auxin in the plants (Meyer *et al.*, 2000). The use of auxins during transformation has also been described to increase the competency of the target plant cell. The amenability of *Arabidopsis thaliana* explants to *Agrobacterium* when pre-cultured on medium containing growth regulators have been reported (Chateau *et al.*, 2000).

In this study, the effect of IBA concentrations in the medium for pre- and co-culture with *A. rhizogenes* were studied. This particular auxin was chosen because in an earlier work elucidating the tissue culture medium for the induction of roots from shoot explants of *E. longifolia*, it was reported that IBA was found most suitable for the induction purpose (Sobri *et al.*, 2005b). Fig. 4.4 shows the transient GUS expression frequency as assayed after treatment at various concentrations of IBA. The highest GUS expression frequency was obtained in explants co-cultivated in medium containing 1.5 mg/L IBA followed by 1.0, 2.0 and 0.0 mg/L IBA. The lowest percentage was obtained for the 0.5 mg/L treatment. There were no

significant differences between 1.0, 1.5 and 2.0 mg/L IBA treatments. Likewise, no differences were detected for explants co-cultivated in 0.0, 1.0 and 2.0 mg/L IBA. Plate 4.14 shows the explants treated at the various concentrations and the most intense staining was observed for the explants treated with 1.5 mg/L IBA. Interestingly, tissues closely resembling hairy root like structures were seen extending from the explants treated with both 1.0 and 1.5 mg/L IBA, as shown in Plate 4.15. However, the use of auxins should be limited to lower concentrations to ensure that 'false positive' roots do not emerge from the explants. Since there was no significant difference between explants treated with 1.0 and 1.5 mg/L IBA, the lower concentration of 1.0 mg/L was chosen for the subsequent optimisation steps.

Plant growth regulators (PGRs) have been reported to activate cell division and dedifferentiation in many tissues. The plant cell cycle and division may be responsible for the efficient incitement of *Agrobacterium* transformation (Chateau *et al.*, 2000). In a study investigating *Agrobacterium*-mediated transformation of *Petunia* mesophyll cells, it was discovered that cells that had not been treated with PGRs could not transiently or stably express the T-DNA encoded *gusA* transgene. The authors (Villemont *et al.*, 1997) concluded that T-DNA could be assimilated into the nucleus and expressed in cells actively undergoing DNA synthesis but not when there is a dearth in cell division. T-DNA integration and stabilisation of transformation call for cell division in the host plant (Karami, 2008).

5.5.3 Dithiothreitol (DTT) concentrations

The protocol known as *Agrobacterium*-mediated transformation is in its essence the exploitation of a pathogen infection process naturally present in the wild. During pathogenic attack, rapid cell death in plants is preceeded by oxidative burst with a quick, transient release of reaction oxygen species (ROS) leading to the hypersensitive response (Greenberg *et al.*, 1994; Wojtaszek, 1997). *In vitro* recalcitrance, defined as the inability of plant tissues to respond to culture manipulations and have been associated to several factors although the exact nature of its cause is unknown (Benson, 2000b). These two common problems are usually the biggest stumbling blocks to achieve a successful transformation in desired plant species. Recent developments are pointing a finger at the production of ROS, both as a normal physiological action as well as a defence reaction towards pathogenic attack (Benson, 2000a). In order to control these responses, the use of antioxidants in plant transformation studies have been widely proposed and utilised in various studies.

Antioxidants were generally divided into two groups based on their putative activities of reducing tissue browning, necrosis and promoting organogenesis while the second group enhances the growth of shoots and roots in different species (Dan, 2008). Due to the nature of the present study, to induce hairy roots (i.e. organogenesis) while simultaneously reducing the widespread tissue browning and necrosis observed in the transformed explants, the first group was selected as the choice antioxidants of which ascorbic acid, citric acid, dithiothreitol (DTT) and polyvinylpyrrolidone (PVP) are members. In a study conducted on *Vitis vinifera* cv. Superior Seedless grape plants were co-cultivated with *Agrobacterium* in a medium

containing 1% PVP and 2.0 mg/L DTT and was found to improve explants viability during and after co-cultivation (Perl *et al.*, 1996). Both these antioxidants were chosen as a combination for the *Agrobacterium*-mediated transformation of *Eurcyoma longifolia* somatic embryos.

The highest GUS positive frequency was achieved for 4.0 mg/L DTT followed by 3.0 and 1.0 mg/L DTT treatments. There are no significant differences between these three concentrations. DTT concentrations of 1.0 and 2.0 mg/L were ranked the lowest in their ability and there is no significant difference between the pair. When the GUS positive explants were viewed (Plate 4.16), explants marked A, D and E corresponding to 1.0, 3.0 and 4.0 mg/L DTT showed the most intense blue staining patterns. DTT is a strong reducing agent that undergoes oxidation in the presence of air and is therefore unstable. It has been suggested that the use of DTT should be minimised unless absolutely necessary. This revelation combined with the erratic GUS frequency results obtained prompted the removal of DTT from the pre- and co-cultivation medium formulation.

5.5.4 Polyvinylpyrrolidone (PVP) concentrations

Similar to the optimisation experiment for DTT, polyvinylpyrrolidone at various concentrations were added to the medium. The highest GUS positive frequency was achieved at 1% PVP while the lowest percentage was obtained by the explants treated with 4% PVP. There were no significant difference between the frequencies observed for 0, 1 and 2% PVP. Likewise there are no significant differences between 0, 2, 3 and 4% PVP treatments. At this juncture the decision had

to be made to maintain or remove PVP from the medium formulation. As mentioned in the previous section, antioxidants are added into the medium to control the defence response initiated by the plant towards the invading *Agrobacterium* cells. This result in extensive browning and necrosis in the transformed explants which has been halted in the somatic embryo explants following the addition of the antioxidants PVP and DTT. The instability of DTT has resulted in its removal from the medium. To do so again with PVP would result in necrosis and browning. Therefore, 1% PVP was maintained in the medium and this gave a GUS positive frequency of 94.2% in the transformed somatic embryo explants that may serve as suitable starting material for further genetic transformation studies.

5.6 Hairy root induction in somatic embryos of *Eurycoma longifolia*

Somatic embryos were transformed with *A. rhizogenes* strain AR12 in co-cultivation medium containing 1.0 mg/L (w/v) IBA and 1% (w/v) PVP, a formula derived from the optimisation experiments. When the explants were washed with cefotaxime and transferred back into fresh medium with the same formulation as above, it was observed that the explants were overgrown with the bacterial strains after several days. Hence, methods to control *A. rhizogenes* growth post-transformation were investigated.

In the first formulation, 1.0 g/L of activated charcoal was added into the medium and compared with medium without charcoal. The bacterial overgrowth was reduced but not completely eliminated in the medium containing activated charcoal. Many substances could be adsorbed on the large inner surface area of activated

charcoal. They are often used to enhance growth and development of plant cells in tissue culture. The irreversible adsorption of inhibitory compounds in the culture medium to the fine network of pores in activated charcoal may have positive effects on morphogenesis as it substantially lowers phenolic exudation, toxic metabolites and brown exudate aggregation. The effect of activated charcoal on growth regulator uptake is still unclear but it is considered that activated charcoal may progressively discharge certain adsorbed products, for example growth regulators and nutrients which then becomes accessible to plants (Thomas, 2008).

The concentration of cefotaxime used was at the highest level recommended and any further increases may be deleterious to the explants. Therefore, a method to enhance the efficiency of the antibiotic without increasing the concentration was required. In a recent study, it was suggested that the addition of silver into antibiotic formulations enhances its efficiency against Gram negative bacteria (Morones-Ramirez *et al.*, 2013) and the use of silver nitrate as an antioxidant in transformation protocols has been reported. Silver nitrate at a concentration of 5.0 mg/L were used in conjunction with 40 mg/L cysteine and 20 mg/L ascorbic acid as an anti-necrotic treatment during the transformation protocol for rice shoot meristem explants. The explants treated with antioxidants produced an average necrotic rate of 6% on each explant area, while those without the treatment had 80% of each explant area producing necrosis (Enríquez-Obregón *et al.*, 1999). Silver nitrate at a concentration of 5.0 mg/L was added to the MS liquid medium containing cefotaxime and used to wash the co-cultivated explants. *A. rhizogenes* overgrowth on the surface of the co-cultivated explants was successfully eliminated. As shown in Plate 4.18, green spots were seen on the somatic embryo explants unlike previous hairy root induction experiments where the explants experienced yellowing and necrosis following the

transformation protocol due to bacterial overgrowth. However, the induction of hairy roots from the transformed somatic embryo explants was not successful.

5.7 Detection of transformants using polymerase chain reaction (PCR)

PCR analysis was conducted on the somatic embryos that were able to survive the rigorous transformation protocol using *Agrobacterium rhizogenes* strain AR12. Genomic DNA from both transformed and untransformed somatic embryos were extracted, purified and the concentrations were determined prior to the screening for *rol* and *vir* genes using PCR.

As shown in Plate 4.19, DNA amplification was achieved for *rolA*, *rolB*, *rolC* and *virD* genes from transformed somatic embryos indicating the successful assimilation of these genes in the host plant. The intensity of the bands was observed to be different with *virD* and *rolA* showing the most and least intense bands respectively. The expected band sizes for each of the genes amplified is 248 bp for *rolA*, 780 bp for *rolB*, 490 for *rolC* and 450 for *virD* and the generated bands corresponds to these expected band sizes, using the DNA marker as an indicator.

The amalgamation of *rolA*, *rolB* and *rolC* loci was considered to be adequate for the initiation of hairy roots, depending upon the plant species and tissue type (Christey, 2001). The efficiency of these genes to produce rapidly growing neoplastic roots is said to be comparable to that of the whole T_L-DNA of Ri plasmid (Spano *et al.*, 1988).

The elucidation of the three *rol* genes using the transposon "loss of function" analysis has been the focus thus far because they seem to be necessary for hairy root induction (White *et al.*, 1985), such that various changes in root morphology and plant phenotype are observed when various *rol* genes are inactived or over-expressed in hairy root cultures.

Delayed senescence, wrinkled green leaves, dwarfism and long internodes was observed in the strikingly abnormal transgenic plants expressing the *rolA* gene (Schmulling *et al.*, 1993). Depending on the strain, the *rolB* gene is approximately 760-830 bp in size and synthesizes a protein consisting of 259-279 amino acids. (Meyer *et al.*, 2000). Suppression of adventitious root induction occurs when there is an over-expression of the *rolB* gene using a constitutive promoter (Spena *et al.*, 1987). A high or low level of expression of *rolB* corresponds with defective growth of these organs and therefore precise levels are needed for the successful initiation of hairy roots (Tanaka *et al.*, 2001).

RolC genes code for 179 to 181 amino acid proteins (Meyer *et al.*, 2000) with transgenic plants expressing the gene exhibited decreased apical dominance and dwarfism. The intricate sequence of governance differs depending upon the presence of complete T-DNA sequences (Schmulling *et al.*, 1988) and ultimately culminates in the induction of shoot meristems, indicating a crucial function in the development of pluripotent stem cells (Gorpenchenko *et al.*, 2006). Defense-related genes such as β-1,3-glucanase are turned on by the expression of the *rolC* gene has been recorded during pathogenesis, suggesting that it has the potential to activate plant defense responses (Kiselev *et al.*, 2006).

VirD2 protein was initially suggested to be involved in the translocation and assimilation of T-DNA into the plant nucleus. However, studies have proven that this is not true although the bipartite nuclear localization signal, situated within the C terminus of the VirD2 protein has been shown to be essential for efficient T-DNA transfer as mutant sequences failed to establish the stable integration of T-DNA sequence (Koukolikova-Nicola *et al.*, 1993).

The presence of *rolA*, *rolB*, *rolC* and *virD* genes in transformed somatic embryo explants of *Eurycoma longifolia* have been conclusively shown by PCR based molecular analysis. However, when the transformed explants were cultured in hairy root induction medium containing activated charcoal, IBA and PVP, no root formation was observed. Any number of reasons could be the factor that prevents the initiation of hairy roots in transformed *Eurycoma longifolia* plants such as the over- or under-expression of *rolB* gene or the initiation of plant pathogenic responses by *rolC*. Further studies are required to determine the actual proof to explain this phenomenon.

CHAPTER 6

CONCLUSION

6.1 Conclusions of research

The establishment of *in vitro Eurycoma longifolia* cultures is a laborious process due to various factors such widespread fungal contaminations and the recalcitrant nature of the medicinal plant *in vitro*. *In vitro* shoots, somatic embryos and plantlet cultures were used for conducting histological and SEM analysis, chemotaxis assays and transformation with four different *Agrobacterium rhizogenes* disarmed strains. Histological analysis and SEM observations revealed that somatic embryos of *Eurycoma longifolia* share similar traits with zygotic embryos. Positive chemotactic responses of *A. rhizogenes* towards somatic embryo explants were detected using swarm plate assay. Hairy root-like structures were seen emerging from *in vitro* stem explants co-cultivated with *A. rhizogenes* strain AR14. However, the number of such explants was too low to be confirmed using GUS assay or PCR based detection of transgenes. Transformation optimisations with somatic embryo explants were conducted by analysing the effect of four different factors on the GUS positive frequency. It was ascertained that AR12 is the most virulent and suitable *A. rhizogenes* strain for transformation of *E. longifolia* somatic embryos. The medium formulation achieved is as follows: Full strength MS medium added with 1.0 mg/L IBA, 1% (w/v) PVP, 3% (w/v) sucrose and solidified with 2.8 g/L Gelrite^{TM}. This medium formulation showed a GUS positive frequency of 94.2%. The stable integration of the *rolA*, *rolB*, *rolC* and *virD* genes from *A. rhizogenes* in the plant

genome was confirmed using PCR-based molecular analysis. When explants co-cultivated in these medium were observed for the expression of hairy roots, none was forthcoming.

6.2 Suggestions for future explorations

The primary research question at the beginning of this study was: Is it possible to transform *Eurycoma longifolia* using *Agrobacterium rhizogenes* strains leading to a successful induction of hairy roots? Through the various experiments conducted, it has been shown that the transformation of *Eurycoma longifolia* is not only possible but is highly efficient as well, as shown by the GUS frequencies observed for the various strains and optimisation factors. However, these frequencies did not translate into the induction of hairy roots in transformed explants. While it is possible to transfer and integrate transgenes into the plant genome, the expression of the genes is governed by mechanisms that are yet unknown. Most of these mechanisms have yet to be discovered due to the lack of concerted effort to elucidate their inner workings and pathways. Therefore, the next research question that ought to be answered is: What are the mechanisms that govern the expression of transgenes in transformed *Eurycoma longifolia* plants? This is the next frontier.

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