IN VITRO PROPAGATION OF BLACK KWAO KEUR

(*Mucuna macrocarpa* Wall.)

THANPITCHA WONGSRIWIWAT

MASTER OF SCIENCE

IN BIOLOGICAL SCIENCES

MAE FAH LUANG UNIVERSITY

2007

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A THESIS SUBMITTED TO MAE FAH LUANG UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF MASTER OF SCIENCE

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ACKNOWLEDGEMENTS

Firstly, I would like to express my sincere gratitude to my advisor, Dr. Sayamrat Panpoom for his kindness, continual guidance and beneficial advice.

My sincere thanks go to my co-advisors, Dr. Chalermpol Kerdmanee, and Associate Professor Yuthana Smitasiri who generously provide me with helpful guidance and suggestion.

I also would like to extend my gratitude to Associate Professor Dr. Pimchai Apavatjrut for her constructive criticism and being my examination committee.

I would like to express my sincere gratitude to Aj. Orapin Riyaprao for her beneficial statistical advice.

I am thankful to Mrs. Jindaporn Boonwattana, Mr. Vichan Moonjoi, Miss Numfon Booncheep for their assistance during my working in the university.

I greatly appreciate my sincere friend, Mr. Tanatorn Saisavoey for his assistance, cooperation, and designing figures in this thesis.

I appreciated the support from Ms. Krongthong Chutima, Luang Anusarnsunthorn Research Fund for Kwao Keur plants throughout my academic career.

Finally, I would like to extend my special gratitude to my family for their constant love and understanding.

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Thesis Title *In Vitro* Propagation of Black Kwao Keur (*Mucuna macrocarpa* Wall.)

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ABSTRACT

Mucuna macrocarpa Wall. (black Kwao Keur) is a Thai medicinal plant species in imminent danger of extinction. Since natural regeneration is poor by seeds and plant growth is very slow. So, asexual propagation is necessary. Protocols for callus induction of *M. macrocarpa* have been established. Murashige and Skoog 1962 (MS) and McCown and Lloyd 1981 (WPM) media were used as basal medium. The internodal explants were cultured on medium supplemented with various combinations of auxins and cytokinins for 4 weeks for callus formation. Creamy compact callus initiated on MS medium and WPM containing 2.0 mg/l α-Naphthaleneacetic acid (NAA) and 1.0 mg/l 6-Benzyl-aminopurine (BAP). The highest growth and size increasing were observed in the callus cultured on MS medium containing 2.0 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1.0 mg/l BAP. Multiplied shoots were induced from nodal explants through forced axillary branching *in vitro*. The explants cultured on the medium containing 1.0 mg/l NAA and 1.5 mg/l BAP exhibited the highest number of developed shoot. However, the frequency of shoot proliferation on media containing BAP alone was relatively low and there were fewer number of shoots in each explant when compare with

those on media the containing NAA and BAP. The shoot multiplication was not observed on medium lacking plant growth regulators. The two to three cm long shoots rooted on both MS medium containing NAA or Indole-3-butyric acid (IBA) alone. The highest number of rooting was markedly enhanced by containing the medium with a combination of 2.0 mg/l NAA. The rooted plantlets were successfully acclimatized for 4 weeks. The regenerated plants did not show any detectable phenotypic variation.

Keywords : Kwao Keur / *Mucuna macrocarpa Wall.* / *in vitro* propagation / plant tissue culture / medicinal woody climber

ชื่อเรื่องวิทยานิพนธ์ การขยายพันธุ์กวาวเครือดำ (*Mucuna macrocarpa* Wall.) ในสภาพปลอดเชื้อ

ผู้เขียน นางสาวธัญพิชชา วงศ์ศรีวิวัฒน์

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อาจารย์ ดร. เฉลิมพล เกิดมณี	กรรมการ
รองศาสตราจารย์ ยุทธนา สมิตะสิริ	กรรมการ

บทคัดย่อ

กวาวเครือดำ (*Mucuna macrocarpa* Wall.) เป็นพืชสมุนไพรของไทยที่มีค่าต่อการศึกษาวิจัย มีความสำคัญทางเศรษฐกิจและมีความเสี่ยงต่อการสูญพันธุ์ การขยายพันธุ์กวาวเครือดำด้วยเมล็ดมักมีข้อจำกัดด้านการพักตัวและการเจริญเติบโตช้า จึงเป็นต้องใช้การขยายพันธุ์กวาวเครือดำในสภาพปลอดเชื้อเข้าช่วย การชักนำแคลลัสกวาวเครือดำสามารถทำได้โดยการใช้ชิ้นส่วนลำต้นอ่อนวางเลี้ยงบนอาหารสูตรพื้นฐาน Murashige และ Skoog (1962) (MS) และ McCown และ Lloyd (1981) (WPM) เติมสารควบคุมการเจริญเติบโตกลุ่มออกซินร่วมกับไซโตไคนินเป็นเวลา 4 สัปดาห์ พบว่าแคลลัสชนิดแข็งสีครีมเกิดบริเวณรอยตัดของชิ้นส่วนลำต้นอ่อนที่วางเลี้ยงบนอาหารทั้งสูตร MS และ WPM เติม NAA ความเข้มข้น 2.0 มก/ล ร่วมกับ BAP ความเข้มข้น 1.0 มก/ล อาหารสูตรที่ส่งเสริมการเจริญเติบโตของแคลลัสและชักนำให้แคลลัสมีขนาดใหญ่ คือ MS เติม 2,4-D ความเข้มข้น 2.0 มก/ล ร่วมกับ BAP ความเข้มข้น 1.0 มก/ล การชักนำการสร้างยอดรวมจากชิ้นส่วนข้อของกวาวเครือดำเกิดขึ้นได้ดีบนอาหารสูตรที่เติม NAA ความเข้มข้น 1.0 มก/ล ร่วมกับ BAP ความเข้มข้น 1.5 มก/ล การชักนำรากของกวาวเครือดำนำยอดที่ได้จากการชักนำในสภาพปลอดเชื้อขนาดความสูง 2-3 ซม. มาวางเลี้ยงในอาหารสูตร MS เติม NAA หรือ IBA พบว่าอาหารสูตร MS เติม NAA ความเข้มข้น 2.0 มก/ล สามารถชักนำให้เกิดรากใน

ปริมาณมาก หลังจากนั้นนำต้นพืชที่สมบูรณ์มาปรับสภาพเป็นเวลา 4 สัปดาห์ ก่อนนำไปปลูกในสภาพธรรมชาติ อย่างไรก็ตามต้นพืชที่ได้จากการเพาะเลี้ยงในสภาพปลอดเชื้อนั้นไม่พบว่ามีการเปลี่ยนแปลงที่แสดงออกทางกายภาพ (phenotypic variation)

คำสำคัญ : เพาะเลี้ยงเนื้อเยื่อ / กวาวเครือดำ / การขยายพันธุ์

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ABBREVIATIONS AND SYMBOLS

2,4-D	=	2,4-Dichlorophenoxyacetic acid
BA	=	N^6-Benzyladenine
BAP	=	6-Benzyl-aminopurine
B5	=	Gamborg (1968)
dw	=	dry weight
GA_3	=	Gibberellic acid
IAA	=	Indole-3-acetic acid
IBA	=	Indole-3-butyric acid
Kinetin	=	6-Furfurylaminopurine
NAA	=	α-Naphthaleneacetic acid
TDZ	=	Thidiazuron
B. superba	=	*Butea superba Roxb.*
P. mirifica	=	*Pueraria mirifica Airy Shaw & Suvatabhandhu.*
M. macrocarpa	=	*Mucuna macrocarpa* Wall.
MS	=	Murashige and Skoog (1962)
WPM	=	McCown and Lloyd (1981) (Woody Plant Medium)
°C	=	degree celsius
h	=	hour
cm	=	centimeter
m	=	meter
mg/l	=	milligram per litre
mm	=	millimeter
rpm	=	revolutions per minute

μM	=	micromolar
ppm	=	part per million
w/v	=	weight/volume
cm^3	=	cubic centimetre

CHAPTER I

INTRODUCTION

1.1 Overview

Mucuna macrocarpa Wall. namely called “black Kwao Keur” in Thai, because of its black fluid inside tuberous roots (Anusarnsunthorn, 1931). It belongs to family Fabaceae. Traditionally, this medicinal plant has long been employed among Thai males for the purposes of tonic effects and preventing erectile dysfunction. It has the strongest medicinal properties of the three kinds; two others that are well known white Kwao Keur (*Pueraria mirifica* Airy Shaw & Suvatabhandhu.) and red Kwao Keur (*Butea superba* Roxb.) (Anusarnsunthorn, 1931). This indigenous Thai herb rarely found; however, we can find a few in northern boundary of Thailand (Anusarnsunthorn, 1931). A species widely distributed through the region in high altitude areas, so the external distribution are Burma, China, and Japan (Wilmot-Dear, 1991). Roots have many types of phytochemical and antioxidative substances that can be used as potential natural medicines (Sang-Arun et al., 2001 and Punsawan, 2003). For this reason the roots have long been popularly used for the wide experimental and clinical trial on its phytohormones potential (Suwansatien, 2001; Sang-Arun et al., 2001; Srijungam et al., 2002; Srijungam et al., 2003; Punsawan, 2003 and Cherdshewasart, et al., 2004). This powerful plant maybe extinct from the forests in the long run, because of large-scale and unrestricted exploitation of this natural resource to meet its ever increasing demand by pharmaceutical industry and numerous experiments and clinical trials coupled with limited cultivation and insufficient attempts for its replenishment, the wild stock of this rare medicinally important plant species has been markedly depleted.

The morphological characteristic of black Kwao Keur (Figure 1.1) are huge climber vine to 70 m in height, a self pollinated tropical legume, trifoliate leaves, leaflets without persistent stipels, racemes with long peduncle, pendulous, two-colored flowers with deep violet winged and greenish tip, unwinged margins fruits and stem-like tuber (Wilmot-Dear, 1991). Natural propagation of *Mucuna* species through seeds is unreliable due to seed coat dormancy and low germination rates (Figure1.2). Conventional propagation through vegetative cuttings is slow and a large number of cuttings do not survive during plantation (Singh, 1991). There is a strong need for an alternative method to produce large number of plants of superior types for conservation and regeneration. In recent years, there has been an increased interest in *in vitro* culture techniques which offer a viable tool for germplasm conservation of rare, endangered, and medicinal plants (Arora and Bhojwani, 1989), (Sharma et al., 1991), (Sudha and Seeni, 1994). Moreover, micropropagation technique is an advanced vegetative propagation technique for producing a large number of genetically uniform and pathogen-free transplants in a limited time and space. Although leguminous tree species are less studied and only a few amenable to *in vitro* cultures (Mehta, 2000), there are a number of reports of *in vitro* propagation of various *Mucuna* species (Wichers et al., 1983; Wichers et al., 1989; Pras et al., 1993; Chattopadhyay et al., 1994 and Chattopadhyay et al., 1995). Unfortunately, there are nowhere preliminary reports on tissue culture studies of *M. macrocarpa* elsewhere. This preliminary research will show great promise in producing clonal planting stocks for woody biomass, afforestation and detailed information regarding this economically medicinal plant for advanced biotechnology.

Figure 1.1 A small branch of *M. macrocarpa Wall.* grew up at Doi Tung, Chiang Rai province, Thailand.

Figure 1.2 The mature seed of *M. macrocarpa Wall***.** that fell to the land of Doi Tung's forest**,** However, it can not germinate in nature.

1.2 Objective of this research

This research was undertaken in order to investigate the completely micropropagated system via callus induction, shoot multiplication, root induction and acclimatization of black Kwao Keur through *in vitro*.

1.3 The research procedures

1.3.1 Plant material and explants preparation

1.3.2 Callus induction

1.3.3 Shoot induction and multiplication

1.3.4 Root induction

1.3.5 Acclimatization

CHAPTER II

LITERATURE REVIEWS

2.1 General characteristics of black Kwao Keur

The genus *Mucuna* ADANSON comprises about 160 species distributed over the area of tropics and subtropics (Chen, 1991), 33 accepted species of climbing vines and shrubs of the family Fabaceae, found worldwide in the woodlands of tropical areas.

Kingdom Plantae
Division Magnolioph
Class Magnoliopsida
Order Fabales
Family Fabaceae (Leguminosae)
Subfamily Faboideae (Papilionoideae)
Genus *Mucuna*
Species *macrocarpa*

Figure 2.1 Taxonomy of black Kwao Keur.

2.2.1 Leaf: very varying sizes, elliptic to ovate (to obovate), acute or shortly acuminate, laterals usually markedly asymmetrical with ratio of widths of abaxial to adaxial 2:1; gently curved; thinly chartaceous to thickly coriaceous, especially on veins; persistent stipels absent (Figure 2.3E).

2.2.2 Inflorescence: arising from old wood, often very long, 5-23 cm, unbranched and bearing 5-17 reduced, knob-like, flower bearing side-branches (secondary axes) spaced

throughout their length but more crowded towards apex; pedicels medium-sized, 8-10 mm long, these and main axis with pubescence like that of the stem but always short and often spreading, and also fine brownish bristles; bracts and bracteoles (Figure 2.1C) all of similar shape, very small but bracteoles larger, ovate, hairy like the axis, bracteoles shorter than calyx (Figure 2.3B, C).

2.2.3 Calyx: hairy like the axis; tube and lobes medium to large, tube broadly cup-shaped, lowest lobe, laterals usually half this length, all narrowly to broadly triangular; upper lip indistinct (Figure 2.1E).

2.2.4 Corolla: large, two-colored, standard pinkish or greenish white or " dirty " white, wings deep violet, keel lighter more reddish-violet with greenish tip; standard 3-3.5 cm long, just over 1/2 keel length, wings 4-5.2 cm long and fairly broad 1.5 cm wide, keel distinctly longer 5-6.3 cm long, margins of standard (Figure 2.1F) and wings conspicuously ciliate in apical region up to 1/4 or 1/3 length (Figure 2.1G).

2.2.5 Fruit: woody, green in living state, 6-15 seeded, very long, markedly laterally flattened 7-10 mm in thickness, linear-oblong with margins often constricted between seeds, straight or slightly curved, broadly acute at apex; surface densely pubescent like the axis (rarely glabrescent with age), ornamented with many irregular longitudinal wrinkles and ridges of varying length and thickness; both margins thickened without central groove, often with irregular woody ridge running along surface close and parallel to margin but without pair of flap-like wings (Figure 2.3D, 2.4 right).

2.2.6 Seed: black, very large, 2.2-3.2 x 1.8-2.8 cm, reniform-discoid and markedly laterally flattened 5-10 mm in thickness; hilum dark brown or black (Figure 2.4 left).

2.2.7 Distribution: *M. macrocarpa* in the northern of Thailand can be found in Doi Chiengdao, and Doi Suthep, Chiang Mai province; besides, the external distribution are Burma, China, and Japan (Figure 2.2).

2.2.8 Habitat: evergreen or mixed forest; often by rivers; 600-1600 m altitude. The species widely distributed through the region in high altitude areas.

Figure 2.2 Details of keys of *Mucuna macrocarpa* Wall. A) stem and leaf B) lateral leaflet C) bud with bract and bracteoles D) inflorescence E) calyx F) standard G) wing H) keel J) pistil K) young fruit (Wilmot-Dear, 1991).

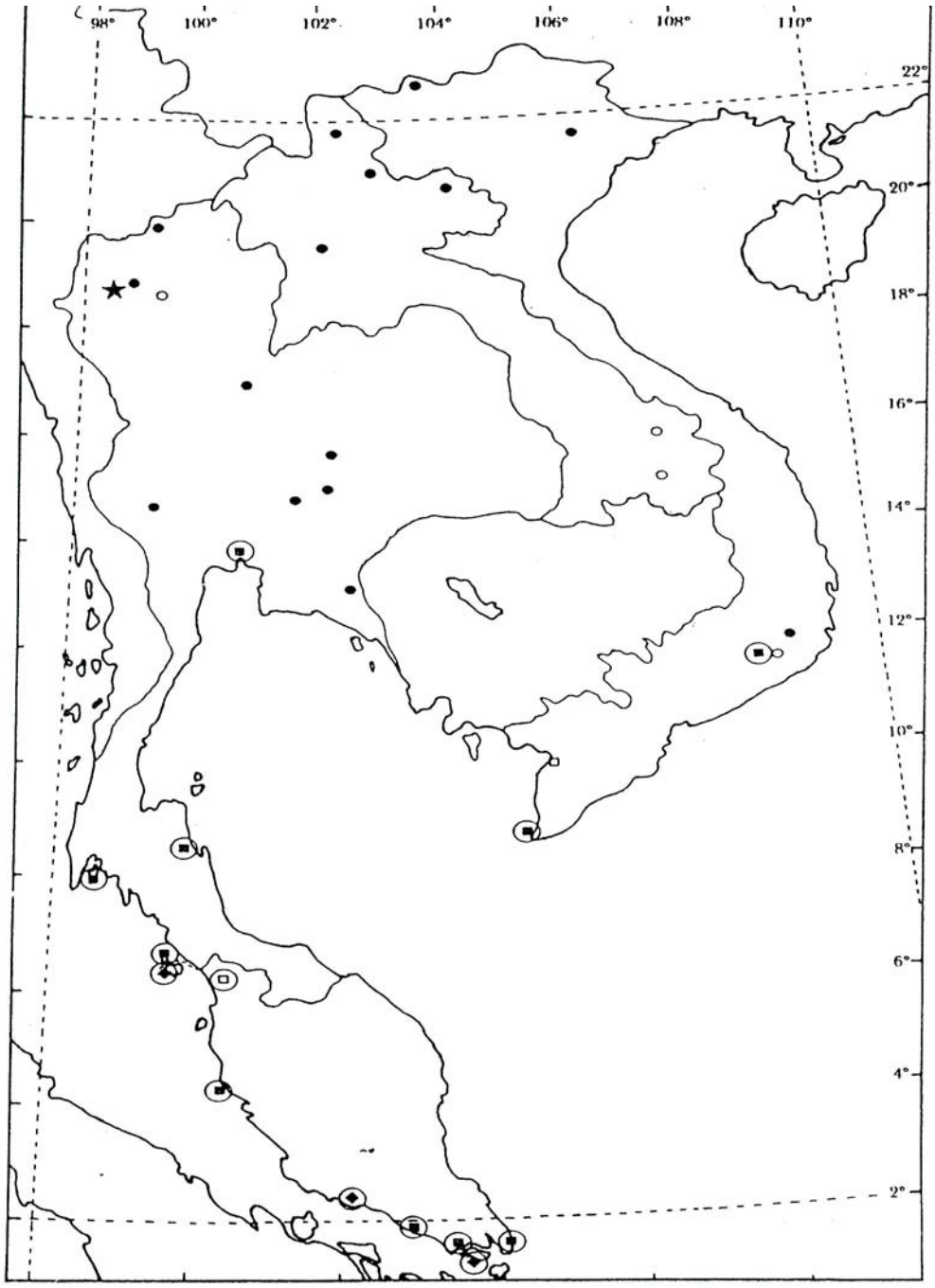

Figure 2.3 Distribution of *Mucuna macrocarpa* Wall. (●) in Thailand, Indochina and the Malay peninsula (Wilmot-Dear, 1991).

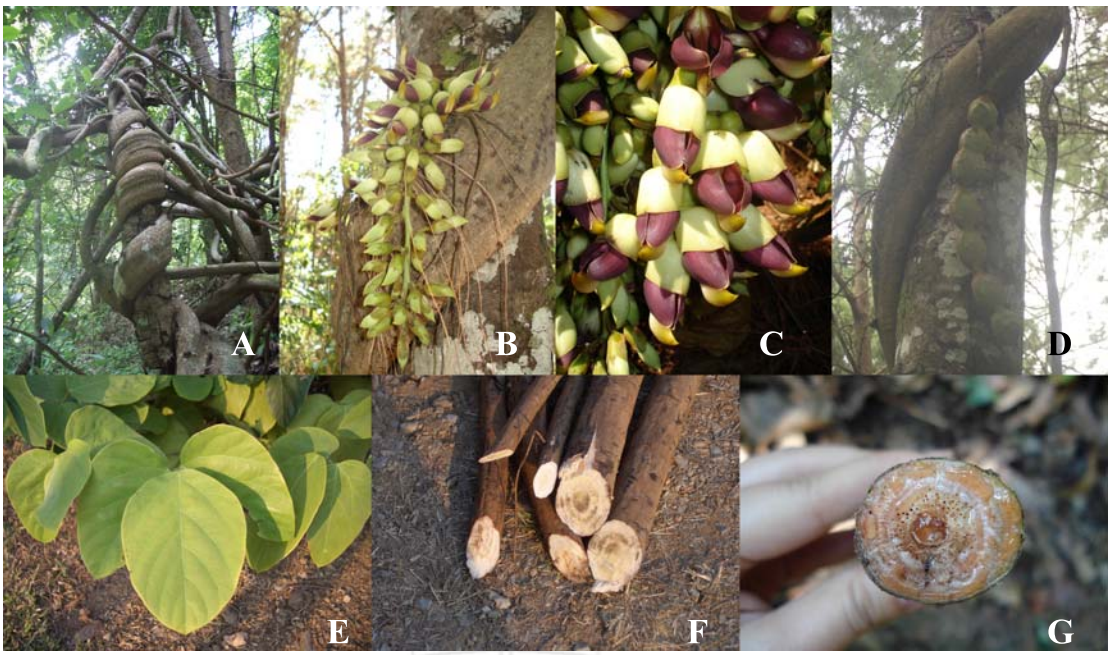

Figure 2.4 Botanical characteristics of *Mucuna macrocarpa* Wall. A) climber stem B) young inflorescence C) mature flowers D) green fruit E) trifoliate leaves F) stem-like tuber G) fluid is exuded from the stem.

Figure 2.5 The black and very hard seed of *M. macrocarpa* (left), the ripened fruit (right). (Bar 1 cm)

2.2 Chemical constituents of *Mucuna*

As Nishihara et al., 2005 said "The allelochemical compound in mucuna has been identified as a non-protein amino acid, L-3-(3,4-dihydroxyphenyl) alanine (L-DOPA). L-DOPA has been shown to be present in mucuna leaves and seeds. The leaves and seeds contain as much as 1% and 4–7% L-DOPA, respectively".

Padmesh et al., 2006 has expressed a similar view that mucunas as a whole are an important source of the toxic compound L-DOPA, hallucinogenic tryptamines and antinutritional factors such as phenols and tannins. Distribution of L-DOPA in the species shows wide variation in different parts of the plant. It is reported to be 0.15 % in dried leaves and pods, 0.49 % in stem and highest concentration is found in raw seed where it ranges from 4.47 to 5.39 %. L-DOPA's physiological mechanism in plants; however, is poorly understood. In animals, L-DOPA is a precursor of the neurotransmitter dopamine and the most effective therapeutic agent for the symptomatic relief of Parkinson's disease. In plants, L-DOPA is a precursor of many alkaloids, catecholamines, flavonoids, melanin, and phenylpropanoids.

2. 3 Experimental trials of *M. macrocarpa*

The work of Cherdshewasart et al., 2004 indicated that black Kwao Keur exhibits only anti-proliferation effects on the growth of MCF-7 cells in relation to a possible anti-estrogen mechanism or a potent cytotoxic effect.

Sang-Arun et al., 2001 concluded that the antioxidant activity of black Kwao Keur is higher than white and red Kwao Keur, but black Kwao Keur is rarely found in nature.

The work of Srijunngam et al., 2002 revealed that a crude extract of *M. macrocarpa* has a reproductive effect in the young tilapia, but the effect is not extended the more mature fish.

From a study by Punsawan, 2003 antioxidative capacity of black Kwao Keur shows that seven solvent were found to be used for black Kwao Keur extraction; Diethyl ether, Dichloromethane, Ethanol, Methanol, Acetone, Acetic acid, and distilled water. Acetic acid was

the best solvent, yielding the highest black Kwao Keur extract weight. Methanol gave a black Kwao Keur extract with highest antioxidative activity.

Suwansatien, 2001 revealed that the acute toxicity of three kinds of Kwao Keur extracts, *P. mirifica*, *M. macrocarpa*, and *B. superba* to the third instar of *Aedes aegypti* larvae and *Culex quinquefasciatus* larvae in the laboratory. This is useful for alternative biopesticides to be used instead of chemical insecticides. The LD_{50} of Kwao Keur extracts from *P. mirifica*, *M. macrocarpa*, *B. superba* to the third instar of *A. aegypti* larvae were 8366, 1873 and 1148 ppm, respectively; and the third instar of *C. quinquefasciatus* larvae were 894, 374, and 740 ppm, respectively.

Saisavoey, 2006 indicated that crude extracts of *Pueraria mirifica* Airy-Shaw & Suvatabandhu, *Butea superba* Roxb. and *Mucuna macrocarpa* Wall. prepared by sequential extraction using three different solvents: hexane, ethyl acetate and methanol, and used to test for their antimicrobial activity using the disc diffusion method. It was showed that only the *P. mirifica* extracts prepared from ethyl acetate exhibited antimicrobial activities against *Bacillus cereus*, *B. subtilis*, *Escherichia coli*, *Micrococcus luteus*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *Saccharomyces cerevisiae*, *Salmonella typhimurium*, *Serratia marcescens*, *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus faecalis* and *S. lactis*.

2.4 *In vitro* culture of related plants

2.4.1 *Mucuna* species

The work of Chattopadhyay et al., 1994 asserted that Production of L-DOPA from cell suspension culture of *Mucuna pruriens f. pruriens* suspension culture was established in MSI medium composed of 1/2 MS salts and sucrose 2%. A two-stage cell suspension culture was developed for enhanced accumulation of L-DOPA. In the first stage, the culture system was composed of MSI medium without $CaCl_2$ which was suitable for cell growth and in the second stage MSI medium containing KH_2PO_4 42.5 mg/l and sucrose 4% favoured L-DOPA production. A discernible higher production of L-DOPA was obtained in this two-stage cell suspension culture in comparison to single stage culture. Initial callus of

M. pruriens (L.) f. pruriens was obtained and maintained following the procedure of Pramanik & Datta, 1986. Cell suspension cultures were obtained from stock callus by placing friable calli (50 mg dw/100 ml medium) in induction medium (MSI) containing 1/2 inorganic salts of MS medium and sucrose 2% (w/v). Suspension cultures were maintained in MSI medium supplemented with sucrose 2% and Kinetin 0.5 mg/l at 120 rpm on a rotatory shaker under continuous illumination. L-DOPA content was measured both from cells and medium.

In an article by Wichers, 1985 investigated that induction of phenoloxidase in cell suspension cultures of *M. pruriens* (L.) DC f. *utilis* (Wall. ex Wight) Back. *cv.* White effects on accumulation of L-3,4-dihydroxyphenylalanine (L-DOPA) and biotransformation capacity. Cell suspension cultures of *M. pruriens* were prepared from calluses, originating from leaf explants, in MS medium supplemented with IAA 1.0 mg/l, BA 1.0 mg/l and sucrose 4% (w/v) at pH 5.9. Nitrogen limitation of the cultures, or the addition of p-Fluorophenylalanine or Ethionine to the culture medium resulted in an increased phenoloxidase activity. There appeared to be an inverse relationship between phenoloxidase activity and the accumulation of L-DOPA. However, the transformation of L-tyrosine into L-DOPA by alginate-entrapped cells occurred at a higher rate when phenoloxidase activity was increased.

2.4.2 Woody plant and leguminous tree

As Simpson, 1997 wrote, “Barker et al., 1997 experimented American Beech with micropropagation of actively growing shoots from seedlings to determine the better of two cultures, Wolter and Skoog (WS) and Aspen Culture (AC) media, and the best combination of the two hormones BA and NAA”.

Quoirin et al., 1998 reported that *in vitro* regeneration of adventitious buds and roots of *Acacia mearnsii* derived from callus obtained from hypocotyl explants that were cultured in 1/2 strength MS medium supplemented with sucrose 2% (w/v), NAA 0.5 mg/l and Kinetin 0.3 mg/l; after that transferred to the MS medium without plant growth regulators for subsequent elongation of shoots.

2.4.3 Callus culture, shoot multiplication and root induction

Paramjit et al., 1990 investigated that differentiation in explants from mature leguminous trees stem and petiole explants, obtained from mature trees, of *Albizzia lebbeck*, *Cassia fistula* and *C. siamea* callused and differentiated shoot-buds and later shoots on B5 medium (Gamborg et al., 1968) supplemented with either IAA 0.5 mg/l combined with 1.0 mg/l BAP or NAA 2.0 mg/l combined with BAP 0.5 mg/l. The stem explants were more responsive than the petiole explants. In *A.lebbeek*, the IAA substituted medium favoured differentiation from both types of explants. However, in *C. fistula*, the type of explants rather than the medium composition had an overriding influence on shoot differentiation since those from petiole hardly responded in either medium. It has been possible to obtain plantlets from both *A. lebbeck* and *C. fistula* under conditions conducive to rooting. Plantlets of *A. lebbeck* have also been successfully transferred to the field.

As Kaur et al., 1998 has indicated, plants obtained from the Khair tree (*Acacia catechu* Willd.) using mature nodal segments, maximum shoot bud development (eight to ten) from a single explant was achieved on MS medium supplemented with BAP 4.0 mg/l and NAA 0.5 mg/l. Addition of adenine sulphate 25.0 mg/l, ascorbic acid 20.0 mg/l and glutamine 150.0 mg/l to the medium was found beneficial for maximum shoot bud induction. The shoot buds developed into healthy and sturdy shoots on MS medium containing BAP and Kinetin at 1.0 mg/l. Excised shoots were rooted on 1/4 strength MS medium with IAA 3.0 mg/l and sucrose 1.5% to obtain complete plants.

Sunnichan et al., 1998 observed that nodal explants from selected trees of Gum Karaya (*Sterculia urens* Roxb.) in the adult growth phase cultured on MS medium supplemented with BAP 1.5 mg/l produced an average of six adventitious shoots in 30 days. Shoots were rooted *in vitro* on 1/4 strength MS medium containing IBA 2.0 mg/l. Nodulated callus was produced from hypocotyl explants cultured on MS medium supplemented with 2, 4-D 1.0 mg/l and BAP 2.0 mg/l.

The work of Pradhan et al., 1998 asserted that cotyledonary nodes derived from 1-week-old axenic seedlings were cultured on MS medium containing either BA, Kinetin, 2iP or TDZ. BA is being the most effective growth regulator. High-frequency shoot proliferation (99%) and maximum number of shoots per explant (7.9 shoots) were recorded with BA 2.0 mg/l at an

optimum level. Concentrations of all cytokinins tested above the optimum level markedly reduced the frequency of shoot proliferation. A proliferating shoot culture was established by repeatedly subculturing the original cotyledonary node on shoot multiplication medium after each harvest of the newly formed shoots. Primary shoots were multiplied as nodal explants, and from each stem node 2 or 3 shoots developed. Thus, 60–70 shoots were obtained in 3 months from a single cotyledonary node. About 91% of the shoots developed roots following transfer to 1/2 strength MS medium containing a combination of IAA 1.0 mg/l, IBA 1.0 mg/l. 80% of the plantlets were successfully acclimatized and established in soil.

In an article by Chengalrayan et al., 1999, a combination of NAA 4.0 mg/l and BAP 5.0 mg/l was optimum for inducing caulogenic buds in *in vitro* regulation of morphogenesis in peanut (*Arachis hypogaea* L.). The caulogenic buds proliferated in a medium with BAP 3.0 mg/l. Differentiation of these buds to shoots was achieved in MS basal medium with each of BAP and Kinetin 0.5 mg/l. Shoot buds and flower buds were produced when caulogenic buds were cultured on the medium containing BAP 1.0 mg/l and Kinetin 1.0 mg/l, prior to elongation. Clonally propagated plantlets derived from axillary buds elongated, formed roots and were grown to maturity in soil. Embryogenic mass formation was induced from the leaf base in the presence of 2,4-D 20.0 mg/l. Somatic embryo developed upon reducing 2,4-D to 3.0 mg/l.

As Almasri, 2000 had indicated that *Podocarpus gracilior* was successfully developed a large number of shoots when cultured on different media supplemented with different concentrations of BAP; however, the best treatment for the production of shoots was modified WPM supplemented with BAP 0.5 mg/l. The most roots were developed on modified WPM supplemented with IBA 0.5 mg/l.

In an article by Mehta, 2000 mature zygotic embryo axis of tamarind (*Tamarindus indica*) consists of longituditional section attached to cotyledons were cultured in a modified MS medium supplemented with NAA 0.5 mg/l, BAP 10.0 mg/l and sucrose 4% (w/v) to induce adventitious shoot buds.

Kulkarni and D'Souza, 2000 reported that *in vitro* shoot tip of *Butea monosperma,* Four to five multiple buds were induced from the axis of the cotyledonary nodes, with cotyledons intact, on 1/2 strength WPM supplemented with BAP 5.0 mg/l alone and sucrose 30 g/l.

A study by Kulneet and Kant, 2000 showed that *in vitro* micropropagation through shoot apices of *Acacia catechu*, explants were excised from 15-day-old *in vitro* grown seedlings raised from superior seed stocks. Shoot bud induction from shoot apex explants was observed on MS medium containing various growth regulators. A maximum of 12 shoots was obtained on MS medium supplemented with BAP 1.5 mg/l and Kinetin 1.5 mg/l. Well-developed shoots (3–4 cm long) were rooted on 1/4 strength MS medium with IAA 3.0 mg/l and sucrose 1.5%.

The work of Sasmitamihardja et al., 2001 revealed that *in vitro* regeneration of *Paraserianthes falcataria* (L.) Nielsen was achieved through axillary shoot multiplication. Single-node segments from six-day-old seedlings were used as explants and cultured on modified MS medium. The addition of IAA 0.01 - 1.0 µM in combination with BA 0.1 - 1.0 µM induced axillary shoot proliferation. During the first two weeks in culture,
4-15 shoots per explant were proliferated. The highest number of shoots was obtained from single-node explants cultured on the medium supplemented with IAA 0.1 µM and BA 0.5 µM. After three weeks in culture, up to 23 shoots per explant were produced. Lengthening the period of culture produced no significant increase in shoot proliferation. In the last week of a 4-week period of culture, the shoot multiplication rate was very low. Regenerated shoots were elongated on MS medium without growth regulators.

Vengadesan et al., 2000 had drawn attention to the fact that many species in the genus *Acacia,* one of the important genera of the family Fabaceae, are more commonly micropropagated by using field explants. Cotyledonary nodes, epicotyl and shoot tip explants were used to induce multiple shoots; however, their data revealed that nodal explants were most suitable for multiple shoot formation. The WPM used commonly for the *in vitro* propagation of woody species had a very little impact on *Acacia* species. MS medium was preferred in many of the *Acacia* species for shoot induction, elongation and root induction. In some cases activated charcoal at 2.0 mg/l was used as an additive to prevent callusing, leaf chlorosis and to improve elongation and rooting. In the genus *Acacia,* frequent subculture of explants at constant intervals (25-30 days) made significant improvement in enhancing the number of multiple shoots.

Liu et al., 2002 reported that *P. lobata* hairy roots have faster elongation and more branches than normal roots. The responses of hairy roots and normal roots to treatment with IAA,

IBA, and NAA were different. In normal roots, all the three auxins strongly stimulated lateral root formation at all tested concentrations. Responses to IAA and IBA in primary root growth and lateral root elongation were similar and depended on concentration; promotion at 0.1 μM, no effect at 1.0 μM, and inhibition at 2.5 μM.

Purohit et al., 2002 pointed out that multiple shoots of *Quercus leucotrichophora* L. and *Q. glauca* Thunb. were induced from the intact embryos (decoated-seeds) as well as from the cotyledonary nodes (with attached cotyledons but without radicle and primary shoot) of 3 weeks old *in vitro* grown seedlings on WPM and MS media supplemented with BA, either alone or in combination with GA_3, IBA. BA at 5.0 mg/l was effective for induction of multiple shoots, and addition of GA_3 to the medium further enhanced the shoot number and shoot height but resulted in shoot thinness. High frequency shoot multiplication was achieved using cotyledonary nodes. Shoots were further multiplied from the original explant on WPM supplemented with BA 5.0 mg/l. Nearly 78% and 67% rooting were obtained in *Q. leucotrichophora* and *Q. glauca* microshoots (3–4 cm high), respectively on 1/2 strength WPM supplemented with 3.0 mg/l IBA. However, this was associated with basal callus formation. Treatment with IBA 5.0 - 20.0 mg/l for 24 or 48 h followed by transferring to plant growth regulator-free 1/2 strength WPM not only improved the rooting percentage but also avoided basal callus formation. IBA at 20.0 mg/l for 24 h was most effective (90% and 100% rooting in *Q. leucotrichophora* and *Q. glauca*, respectively). *In vitro* rooted plants were hardened and established in garden soil.

Al-Wasel, 2002 reported that *in vitro* mass propagation of *Acacia seyal* using seedling shoot tip explants was achieved on MS medium supplemented with BA in the presence of NAA. The greatest shoot multiplication with long shoots was observed on the media containing BA 2.0 mg/l with NAA 0.1 or 0.5 mg/l and BA 4.0 mg/l with NAA 0.1 mg/l; with mean number of shoots at 6.4 and 6.7, respectively. Microshoots were rooted better on 1/2 MS medium supplemented with IBA. The highest rooting percentage (80%) and root number (4.9 roots / microshoot) were promoted by IBA 4.0 mg/l. The plantlets (90%) successfully survived acclimatization *ex vitro.*

The work of Thakur et al., 2002 asserted that a callus was derived from cultured cotyledons on MS medium supplemented with 2,4-D 0.25 mg/l and NAA 0.25 mg/l. Plantlets regenerated from the callus and nodal explants on MS medium containing BAP 2.0 mg/l and Kinetin 2.0 mg/l were further multiplied on the same medium. Addition of adenine sulphate 25.0 mg/l, ascorbic acid 20.0 mg/l and glutamine 150.0 mg/l in the medium resulted in enhanced axillary branching. Multiple shoots formed after 6 weeks were separated and subcultured in the fresh medium of same composition. For rhizogenesis, microshoots of 2.0-2.5 cm in length were dipped in sterilized IAA 10 mg/l solution for 24 h followed by transferring to half strength MS medium containing activated charcoal (0.02%), resulting in rooting (75%) within 8 weeks. The rooted plants were transferred to pots containing sterilized soil and sand mixture for hardening and 71% survival was recorded. Fifty true-to-type plantlets of *A. catechu* could be obtained within seven months of culture establishment.

A study by Nanda, 2003 showed that from *in vitro* propagation of *Acacia mangium,* a tropical leguminous tree, bud sprout was obtained from mature nodal explants of 10-year-old tree on MS basal medium supplemented with BAP 1.0 mg/l, $GA_3$1.0 mg/l and IAA 0.05 mg/l. The rate of multiplication was obtained on MS medium supplemented with BAP 1.5 mg/l, IAA 0.05 mg/l and adenine sulfate 100 mg/l. The multiplication rate varied from 1 to 8, depending on the plant growth regulators used. Excised shoots were rooted on 1/2 strength MS basal salts supplemented with IBA 0.5 mg/l or IAA and sucrose 20 g/l (w/v) after 13-14 days of culture.

Thiem, 2003 indicated that i*n vitro* clonal propagation of a valuable medicinal plant, i.e. *P. lobata* by enhancing axillary bud proliferation in shoot tip explants achieved on MS medium supplemented with Kinetin 1.0 mg/l and IAA 1.0 mg/l, yielding an optimum frequency of shoot formation (94%) and shoot number (3.6 shoots per explant). The best shoot elongation was obtained on the MS medium containing GA_3 2.0 mg/l and BA 1.0 mg/l. Rooting was the highest (100%) on the full-strength MS medium with NAA 0.5 mg/l, IAA 0.5 or 2.0 mg/l.

According to Minh 2005, *Aquilaria crassa* (agarwood), a Vietnamese forest tree, was micropropagated using shoot explants from 20-year-old trees known to produce the valuable exudates ‘tok’. Either shoot tips or internodes could be used for the initial explants, but in subcultures best results were obtained from internodes. WPM was a better basal medium than MS, and for initial shoot induction BA 1.0 mg/l and coconut water at 10% (v/v) were used. For

subcultures, BA 0.1 mg/l, NAA 0.1 mg/l and coconut water at 10% gave highest shoot multiplication. A low level of rooting was obtained using either IBA or NAA at 0.3 mg/l. Plants transferred to the field grew to 2 m after 18 months and had normal morphology.

According to Lyyra et al., 2006, *in vitro* regeneration of black willow plants (*Salix nigra* Marsh.) could be obtained by using unexpanded inflorescence explants excised from dormant buds. The highest shoot regeneration frequency (36%) was achieved on BAP 0.5 mg/l. Mean number of shoots per explant varied from one to five. The ability of black willow inflorescences to produce adventitious shoots makes them potential targets for *Agrobacterium*-mediated transformation with heavy-metal-resistant genes for phytoremediation.

Kartsonas and Papafotiou, 2007 discovered that using nodal explants from seedlings in micropropagation of *Quercus euboica* gave higher multiplication rates than explants from adult plants. WPM salts, with myoinositol 100 mg/l, thiamine 1.0 mg/l, pyridoxine 0.5 mg/l, nicotinic acid 0.5 mg/l and sucrose 3% was used as basal medium and several cytokinins at various concentrations were evaluated for their effect on shoot multiplication. The highest shoot multiplication rate was obtained from BA 1.0 mg/l. But IBA 2.0 mg/l in the culture medium during the first week of culture, and followed by culturing in a plant growth regulator-free medium, gave the best rooting results. Darkness at the beginning of the rooting period did not improve rooting. The use of plastic wrap as a cover material for the culture vessels enhanced rooting percentage and root number. Plantlets acclimatized *ex vitro* in soil from the natural environment where the species grow survived at a higher percentage (up to 93%) and had more vigorous growth than those grown in a compost–perlite (2:1 v/v) medium (only 36%).

CHAPTER III

RESEARCH METHODOLOGY

3.1 Materials and Equipments

3.1.1 Seeds from Doi Tung, Chiangrai province, Thailand

3.1.2 Stock solutions of Murashige and Skoog (1962) (MS)

3.1.3 Stock solutions of McCown and Lloyd (1981) (WPM)

3.1.4 Plant growth regulators

1. Auxins
    1) α-Naphthaleneacetic acid (NAA)
    2) 2,4-Dichlorophenoxyacetic acid (2,4-D)
    3) Indole-3-butyric acid (IBA)
2. Cytokinins
    1) 6-Furfurylaminopurine (Kinetin)
    2) 6-Benzyl-aminopurine (BAP)

3.1.5 Buffers for pH meter

1. HCl 1 N
2. NaOH 1 N

3.1.6 Sterilants

1. 70 % ethanol
2. 95 % ethanol
3. Clorox™ (NaOCl 6.00%)

3.1.7 Tween-20®

3.1.8 Mild detergent: Teepol™

3.1.9 Alcohol lamp

3.1.10 Culture vessels: wide necked bottle with fitted lids sized 8 onz.

3.1.11 Handling pipette and sterile pipette tip

3.1.12 Weighing balance 1 to 200 g

3.1.13 Weighing balance 1.0-0001 g

3.1.14 pH-meter

3.1.15 Hot plate with magnetic stirrer

3.1.16 Autoclave

3.1.17 Sterilized forceps (18 cm long)

3.1.18 Sterilized scalpels (15 cm long) and blades

3.1.19 Laminar flow cabinet

3.1.20 Glassware-Duran bottles with rims, glass cylinders, beakers

3.1.21 Glass rod stirrers

3.1.22 Sterilized petri dishes

3.1.23 Gyratory shaker

3.1.24 Sterilized rack (autoclaved)

3.1.25 Plastic syringes sized 20, 60 cc.

3.1.26 Hot air oven for drying glassware

3.1.27 Microwave oven

3.1.28 Paper towel

3.1.29 Trolley for carrying hot media flasks and containers

3.1.30 Aluminum foil

3.1.31 Refrigerator

3.1.32 Sterilized distilled water (autoclaved)

3.1.33 Cool white fluorescent light source

3.1.34 Spray bottle

3.2 Plant material and explant preparation

Uniform and uncracked seeds of the *M. macrocarpa* were collected from Doi Tung, Chiangrai province, Thailand as source of explants (Figure 3.1A). The seeds were pretreated germination for breaking seed coat dormancy (Singh et al., 1991), followed by an extensive water rinse in running tap water for 30-45 min, surface sterilized by immersing in 25% (v/v) Clorox™ (NaOCl 6.00%) containing 2 to 3 drops Tween-20® as a wetting agent for 30 min with continuous shaking, and finally rinsed with distilled water for 10 min three times aseptic conditions to remove the trace of sterilant. Surface-sterilized seeds were cultivated on plant growth regulator-free MS (Murashige and Skooge, 1962) solidified medium with 0.245% (w/v) Phytagel™ (Sigma Chemical, St. Louis, USA) containing 3% (w/v) sucrose (Figure 3.1B).

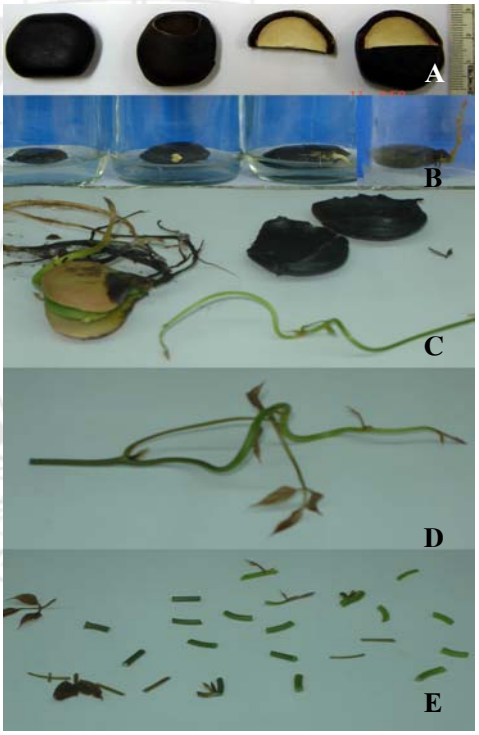

Figure 3.1 A) Uniform seeds of the *M. macrocarpa* from Doi Tung, Chiangrai province, Thailand B) seeds germination *in vitro* C) excised explants from seedling in aseptic condition D) excised shoot E) explants were cut into 1 cm segments.

The pH of the medium was adjusted to 5.8 before steam sterilized at 121 °C for 20 min. The pretreated seeds were maintained at low light intensity, 25±2 °C temperature and 50 - 60 % humidity. Ten to fourteen days after seedling emergence, explants were cut 1.0 cm long segments and placed into culture medium (Figure 3.1E, 3.2). During initial subculturing, the mother explants were kept intact with proliferated shoots.

3.3 Culture media and condition

3.3.1 Callus induction

1. callus induction experiment 1: The culture media used for this study were MS media and WPM variously supplemented with different concentrations and combinations of auxins and cytokinins (Table 3.1) in 2x2x3 factorial in completely randomized design. Results were compared with plant growth regulator-free media (MS and WPM basal medium; control treatments). So, this experiment have 20 treatments; 15 replicas per treatment. The internodal explants were placed on that media. Callus morphology and color were used for module recorded after 4 weeks of culture (Figure 3.3, Table 3.3)

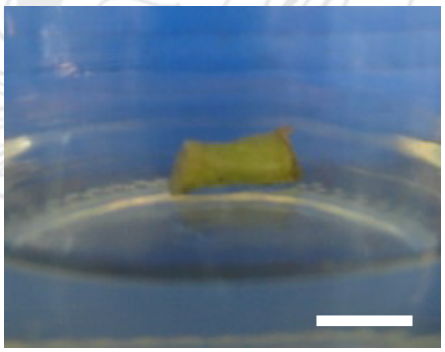

Figure 3.2 The excised internodal explant 1 cm long segment were placed on the medium for callus induction (Bar 1 cm).

Table 3.1 Components of media for callus induction; MS medium and WPM supplemented with variously concentration of NAA and BAP (experiment 1).

Treatment	Components (mg/l)
C1	MS + 0 NAA + 0 BAP
C2	MS + 0.5 NAA + 0.5 BAP
C3	MS + 0.5 NAA + 1.0 BAP
C4	MS + 0.5 NAA + 2.0 BAP
C5	MS + 1.0 NAA + 0.5 BAP
C6	MS + 1.0 NAA + 1.0 BAP
C7	MS + 1.0 NAA + 2.0 BAP
C8	MS + 2.0 NAA + 0.5 BAP
C9	MS + 2.0 NAA + 1.0 BAP
C10	MS + 2.0 NAA + 2.0 BAP
C11	WPM + 0 NAA + 0 BAP
C12	WPM + 0.5 NAA + 0.5 BAP
C13	WPM + 0.5 NAA + 1.0 BAP
C14	WPM + 0.5 NAA + 2.0 BAP
C15	WPM + 1.0 NAA + 0.5 BAP
C16	WPM + 1.0 NAA + 1.0 BAP
C17	WPM + 1.0 NAA + 2.0 BAP
C18	WPM + 2.0 NAA + 0.5 BAP
C19	WPM + 2.0 NAA + 1.0 BAP
C20	WPM + 2.0 NAA + 2.0 BAP

2. Callus induction experiment 2: The culture media used for this study were MS medium variously supplemented with different concentrations and combinations of both auxins and cytokinins (2,4-D, BAP and Kinetin) or auxin alone (Table 3.2) in 3x2 factorial in completely randomized design. Results were compared with plant growth regulator-free (MS and WPM basal medium; control treatments). So, this experiment have 20 treatments; 15 replicas per treatment. The internodal explants were placed on that media. Callus morphology and color were used for module recorded after 4 weeks of culture (Figure 3.3, Table 3.3)

Table 3.2 Components of media for callus induction, MS medium supplemented with variously concentration of 2,4-D combined with BAP and Kinetin and alternative 2,4-D for callus induction (experiment 2).

Treatment	Components (mg/l)
K1	MS + 1.0 2,4-D + 1.0 BAP
K2	MS + 2.0 2,4-D + 1.0 BAP
K3	MS + 4.0 2,4-D + 1.0 BAP
K4	MS + 1.0 2,4-D + 1.0 Kinetin
K5	MS + 2.0 2,4-D + 1.0 Kinetin
K6	MS + 4.0 2,4-D + 1.0 Kinetin
K7	MS + 0.5 2,4-D
K8	MS + 1.0 2,4-D
K9	MS + 1.5 2,4-D
K10	MS + 2.0 2,4-D
K11	MS + 2.5 2,4-D
K12	MS + 3.0 2,4-D

The callus formation was scored by after 4 weeks of culture. The score modules were determined using color and morphological characteristics of the callus subjectively.

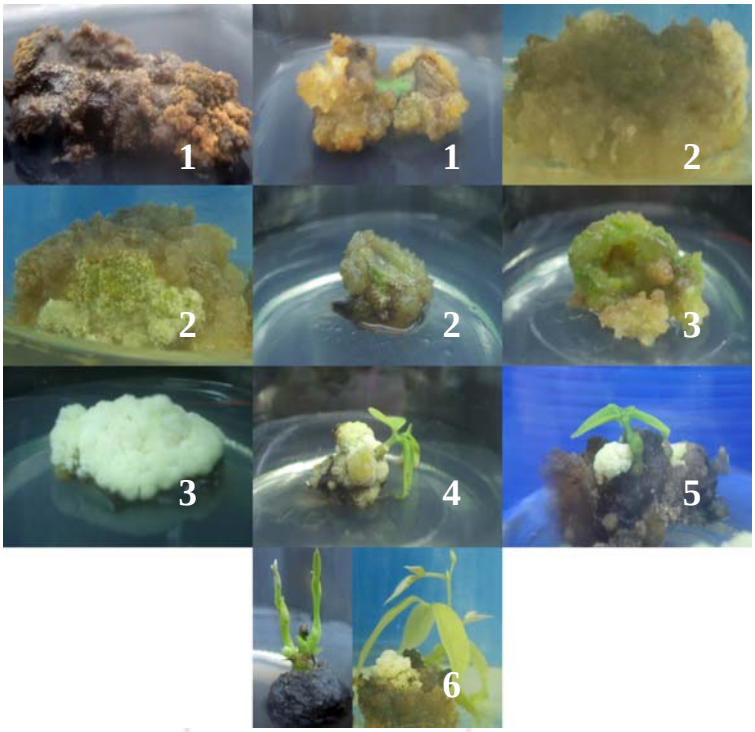

Figure 3.3 The examples of color and friability of calli were used to scores' modules for callus induction**.** The number in each figure is the score of callus formation.

Table 3.3 The morphological characteristics of callus derived from the internodal explants were used to score for determining the callus induction.

Score level	Modules (color and friability)
0	Non callus initiation
1	Dark callus/ brownish callus/ friable/ compact
2	Yellowish white/ yellowish gray callus/ friable
3	Yellowish green callus/ friable
4	Hard creamy callus/ compact
5	Hard creamy callus/ compact with a shoot
6	Friable/ compact callus with shoots

3.3.2 Shoot multiplication

1. Shoot multiplication experiment 1: The culture media used for this study were MS medium variously supplemented with alternative BAP in completely randomized design. Results were compared with plant growth regulator-free MS media (control treatments). So, this experiment have 5 treatments; 15 replicas per treatment. The nodal explants were placed on that media. Numbers of shoot per explant was recorded after 4 weeks of culture.

Table 3.4 Components of media for shoot multiplication; MS medium supplemented with variously concentration of alternative BAP (Experiment 1).

Treatment	Components (mg/l)
S1	MS + 0 BAP
S2	MS + 0.5 BAP
S3	MS + 1.0 BAP
S4	MS + 1.5 BAP
S5	MS + 2.0 BAP

2. Shoot multiplication experiment 2: The culture media used for this study were MS and WPM medium variously supplemented with different concentrations of NAA and BAP in 2x3x3 factorial in completely randomized design. Results were compared with plant growth regulator-free MS and WPM media (control treatments). So, this experiment have 20 treatments; 15 replicas per treatment. The nodal explants were placed on that media. Numbers of shoot per explant and shoot length were recorded after 4 weeks of culture.

Table 3.5 Components of media for shoot multiplication; MS media and WPM supplemented with variously concentration of NAA and BAP (experiment 2).

Treatment	Components (mg/l)
H1	MS + 0 NAA + 0 BAP
H2	MS + 0.1 NAA + 0.5 BAP
H3	MS + 0.1 NAA + 1.0 BAP
H4	MS + 0.1 NAA + 1.5 BAP
H5	MS + 0.5 NAA + 0.5 BAP
H6	MS + 0.5 NAA + 1.0 BAP
H7	MS + 0.5 NAA + 1.5 BAP
H8	MS + 1.0 NAA + 0.5 BAP
H9	MS + 1.0 NAA + 1.0 BAP
H10	MS + 1.0 NAA + 1.5 BAP
H11	WPM + 0 NAA + 0 BAP
H12	WPM + 0.1 NAA + 0.5 BAP
H13	WPM + 0.1 NAA + 1.0 BAP
H14	WPM + 0.1 NAA + 1.5 BAP
H15	WPM + 0.5 NAA + 0.5 BAP
H16	WPM + 0.5 NAA + 1.0 BAP
H17	WPM + 0.5 NAA + 1.5 BAP
H18	WPM + 1.0 NAA + 0.5 BAP
H19	WPM + 1.0 NAA + 1.0 BAP
H20	WPM + 1.0 NAA + 1.5 BAP

3.3.3 Root induction

Rooting of microshoots (2-3 cm long) are tested by subculturing them on MS media containing different types and levels of auxins (NAA and IBA) apply alternatively at various concentrations in completely randomized design. Results were compared with plant growth regulator-free MS media (control treatments). So, this experiment have 9 treatments; 15 replicas per treatment. Numbers of root per shoot was recorded after 4 weeks of culture.

Table 3.6 Components of media for root induction from the microshoot; MS medium supplemented with variously concentration of alternative NAA and IBA.

Treatment	Components (mg/l)
R1	MS + 0 NAA
R2	MS + 0.5 NAA
R3	MS + 1.0 NAA
R4	MS + 1.5 NAA
R5	MS + 2.0 NAA
R6	MS + 0.5 IBA
R7	MS + 1.0 IBA
R8	MS + 1.5 IBA
R9	MS + 2.0 IBA

3.3.4 Media and condition

All of modified MS media supplemented with 3% (w/v) sucrose and modified WPM with 2% (w/v) sucrose. The pH of the MS and WPM media was adjusted to 5.8 and 5.6, respectively using 1 N NaOH or 1 N HCl before gelling them with 0.245% Phytagel™ (Sigma) prior to autoclaving (121°C, 20 min). 30 ml of medium are dispensed into culture vessels. The cultures are maintained at 25 $\pm$ 2°C under cool white fluorescent lamps (about 2,000 lux) in a photoperiod of 16 h for a day and with 55 - 60 % relative.

3.3.5 Acclimatization and transfer of plantlets to soil

Plantlets with well-developed roots were removed from the culture medium and after washing the roots gently with sterile distilled water under *in vitro* condition, plantlets were transferred to sterile vermiculite supplemented with sucrose-free MS liquid medium for 4 weeks. After that loosen the plastic cap for a week. plastic pots (13 cm diameter) containing autoclaved garden soil. The relative humidity was reduced gradually and after 30 days the plantlets were transplanted to earthenware pots (25 cm diameter) containing garden soil and kept under shade in a net house for another 2 weeks before transferring outside under full sun.

3.4 Data analysis of the experiments

The data were analyzed using analysis of variance (ANOVA) with a significantly confidence limit of 0.05 and 0.01 level. Differences between the means are compared by Tukey's test.

CHAPTER IV

RESULTS AND DISCUSSION

This preliminary research chooses newly germinated seedlings as a choice of explant material since seeds are relatively easy to sterilize and can be germinated and grown under aseptic conditions. However, it is important to bear in mind that the seeds of *Mucuna* species have the typical hard seed coats, which must be scarified for moisture uptake and timely germination (Heit, 1967). Singh et al., 1991 revealed that in North America, scarification is commonly done with concentrated sulfuric acid, both for nursery plantings and for laboratory germination tests. Recommended times for acid treatment range from 1/2 to 3 hours. Seed coat thickness is quite variable, especially in *Robinia pseudoacacia* and *Gleditsia triacanthos*, so time trials with small samples are always advisable before treating large lots (Heit, 1967). After the seeds were collected from Doi Tung, Chiangrai province, dried indoor for 2 to 3 weeks, cleaned and stored at 4 °C until the tests began. Then the seeds were soaked with sulfuric acid for 3 h, followed by an extensive water rinse. This pretreatment gave the optimal germination than non treatment seeds (data not shown).

4.1 Callus induction

Internodal segments were cultured on MS and WPM media supplemented with various concentration of NAA combined with BAP in the experiment 1 and MS medium supplemented with 2,4-D combined with BAP or Kinetin and alternative 2,4-D in the experiment 2. Within 4 weeks of inoculation in media supplemented with varied plant growth regulators, callus was initiated from cut edge of the explants, on which 100% of the explants formed callus. In the callus induction experiment 1, all of 20 compositions of NAA and BAP tested with the

concentration ranged from 0 up to 2.0 mg/l, callus was initiated only on 18 media that supplemented with varied plant growth regulators, both plant growth regulator-free MS media and WPM failed to induce callus in this study. It can be postulated that plant growth regulators are essential to callus induction. Morphological variation of the induced calluses was observed and they were categorized as follows:

Type I calluses were soft, yellowish white or yellowish gray in color with a fluffy, wet looking surface, easy to break into small pieces when touched. They were formed on both MS and WPM medium supplemented with 0.5, 2.0 mg/l NAA combined with 0.5, 1.0 mg/l BAP. However, the growth of calluses was rather slow while the MS medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP combinations gave a good growth rate (Figure 4.2). All above the MS media supplemented with 2,4-D individually, either 2,4-D with BAP or Kinetin were induced these callus type (Figure 4.1, J-K).

Type II calluses were yellowish green in color friable with a nodular. They were induced well on above all of the medium varied NAA and BAP concentration (Figure 4.1, G-H-I).

Type III calluses were greenish creamy in color with a nodular compact. They were formed on the MS medium with 1.0 mg/l NAA plus 2.0 mg/l BAP. However, the growth of calluses was rather slow compare with Type I and Type II (Figure 4.1, B-C-L).

Type IV calluses were yellowish creamy in color, hard compact, smooth globular structures, bearing high competent of organogenesis. Calluses of this type were induced on the medium containing 2.0 mg/l NAA in combination with BAP at 2.0 mg/l (Figure 4.1, A).

Type V calluses were dark brown or yellowish brown in color with a nodular friable or compact. They did not change their texture, grew very slowly, became browning and died after 8^{th} week of culture period. They were formed on the media with high concentration of 2,4-D (Figure 4.1, D-E-F).

The highest score of callus (5.73 ± 0.12) were record at MS medium supplemented with 2.0 mg/l NAA and BAP (Table 4.1). BAP concentration increasing from 0.5 to 2.0 mg/l resulted in an increase in the rate of callus formation ability. It is the same to the earlier reports where the higher concentration of BAP combination with auxin enhanced the callus formation (Vengadesan, 2000).

Maximum callusing response was observed in the medium supplemented with 2.0 mg/l 2,4-D and 1.0 mg/l BAP (Table 4.3). However, above all callus obtained from 2,4-D and BAP combinations were yellowish gray friable at the initial stage and gradually turned to browning callus form (Figure 4.4B). Above all yellowish gray friable callus were initiated on MS medium supplemented with either 2,4-D with BAP or Kinetin or alone (Figure 4.5). Increasing the concentration of 2,4-D from 2.0 to 3.0 mg/l resulted in a decrease in the rate of compact callus formation. It is in contrast to the earlier reports where the higher concentration enhanced the compact callus formation in genus *Acacia* (Vengadesan, 2000). The callus when subcultured onto the MS medium containing different concentrations of NAA and BAP revealed that there was no regeneration response (data not shown). It can be postulated that the concentrations of NAA and BAP in this study were too low or high to regenerate shoot. The combination of 2,4-D and Kinetin produced yellowish gray and friable and non-regenerative callus.

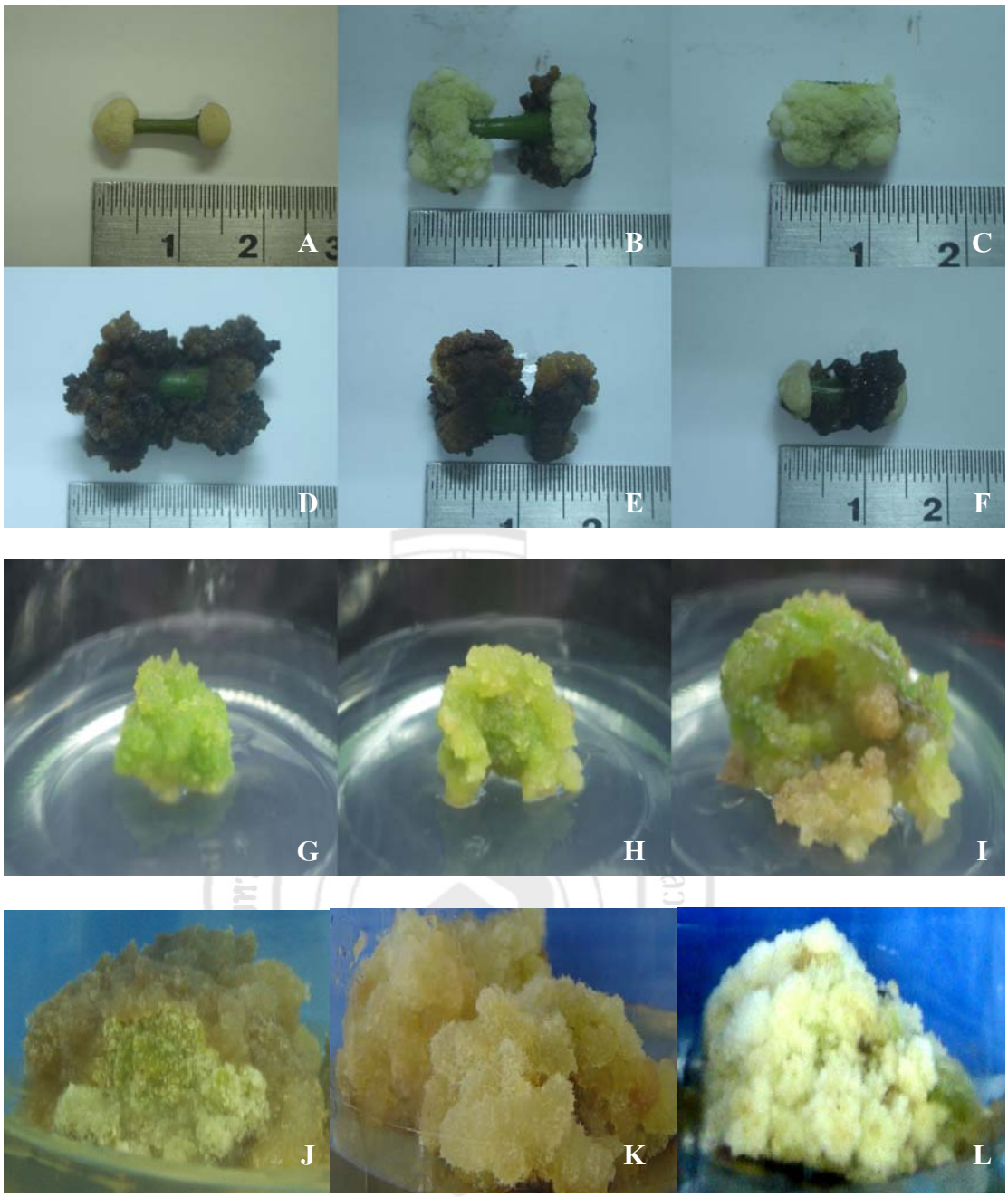

Figure 4.1 Morphology and growth of the callus varied with different level of NAA combined with BAP and 2,4-D individually, either 2,4-D with BAP or Kinetin. Callus type I (J, K) Callus type II (G, H, I) Callus type III (B, C, L) Callus type IV (A) Callus type V (D, E, F).

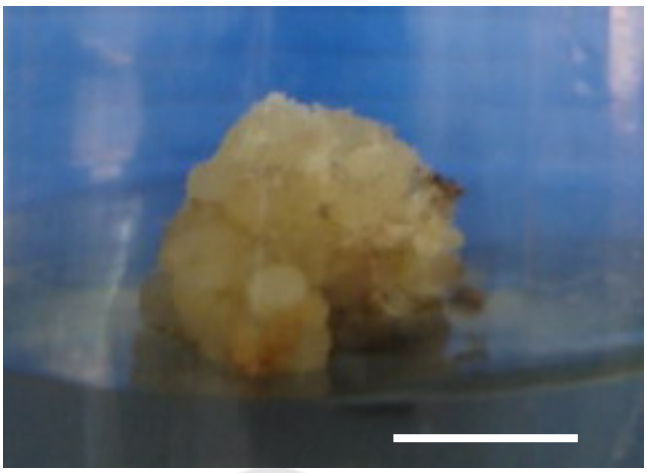

Figure 4.2 Yellowish gray friable callus in the medium containing 2,4-D 2.0 mg/l and BAP 1.0 mg/l (Bar 1 cm).

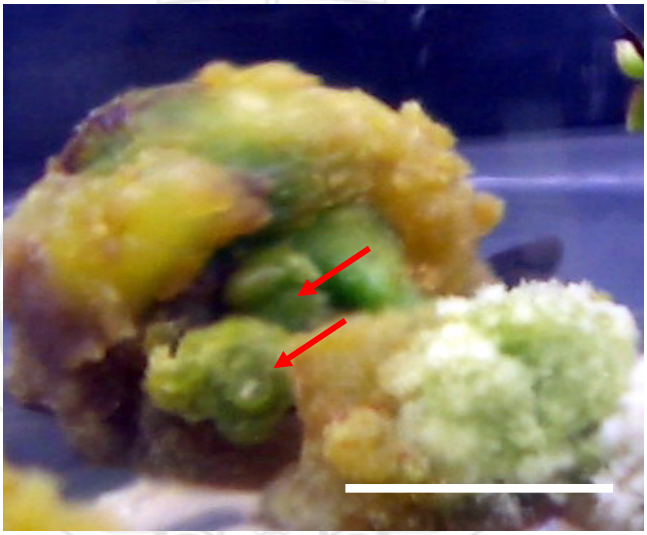

Figure 4.3 Adventitious shoot buds differentiated from the surface of the Type IV callus within 4 weeks of culture (Bar 1 cm).

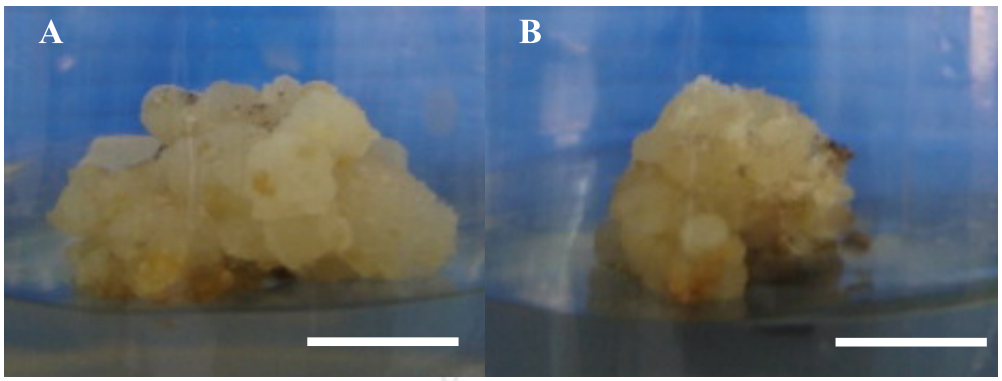

Figure 4.4 (A), (B) Type I of callus obtained from MS medium supplemented with 2,4-D individually, 2,4-D with BAP or Kinetin (Bar 1 cm).

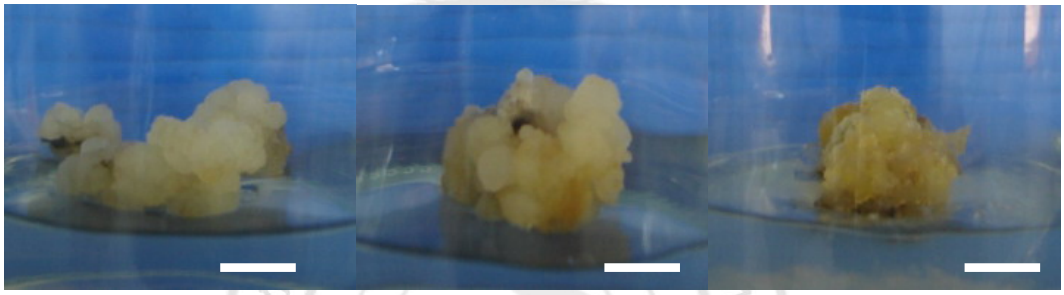

Figure 4.5 (A), (B), (C) Yellowish gray, loose friable calluses were initiated on MS medium supplemented with 2,4-D individually and combination with BAP or Kinetin (Bar 1 cm).

Table 4.1 Effects of MS medium supplemented with NAA and BAP on callus induction scores from internodal explants of *M. macrocarpa* after 4 weeks of culture.

Composition (mg/l)	Mean score of callus
MS + 0 NAA + 0 BAP	1.00 ± 0.00^{a}
MS + 0.5 NAA + 0.5 BAP	1.80 ± 0.11^{b}
MS + 0.5 NAA + 1.0 BAP	2.87 ± 0.13^{c}
MS + 0.5 NAA + 2.0 BAP	3.40 ± 0.13^{c}
MS + 1.0 NAA + 0.5 BAP	3.47 ± 0.13^{d}
MS + 1.0 NAA + 1.0 BAP	3.40 ± 0.13^{d}
MS + 1.0 NAA + 2.0 BAP	3.53 ± 0.13^{d}
MS + 2.0 NAA + 0.5 BAP	1.87 ± 0.09^{b}
MS + 2.0 NAA + 1.0 BAP	2.73 ± 0.15^{b}
MS + 2.0 NAA + 2.0 BAP	5.73 ± 0.12^{e}
F-test	**
Grand mean	2.91
CV	15.90%

Values represent mean $\pm$ standard error of fifteen replicates per treatment. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.01 probability level.

** Strongly significant at 0.01 probability level.

Table 4.2 Effects of WPM medium supplemented with NAA and BAP on callus induction scores from internodal explants of *M. macrocarpa* after 4 weeks of culture.

Composition (mg/l)	Mean score of callus
WPM + 0 NAA + 0 BAP	1.00 ± 0.00^{a}
WPM + 0.5 NAA + 0.5 BAP	1.67 ± 0.13^{b}
WPM + 0.5 NAA + 1.0 BAP	2.67 ± 0.13^{c}
WPM + 0.5 NAA + 2.0 BAP	3.27 ± 0.12^{c}
WPM + 1.0 NAA + 0.5 BAP	3.40 ± 0.13^{d}
WPM + 1.0 NAA + 1.0 BAP	3.33 ± 0.13^{d}
WPM + 1.0 NAA + 2.0 BAP	3.40 ± 0.13^{d}
WPM + 2.0 NAA + 0.5 BAP	1.80 ± 0.11^{b}
WPM + 2.0 NAA + 1.0 BAP	2.33 ± 0.13^{b}
WPM + 2.0 NAA + 2.0 BAP	5.47 ± 0.13^{e}
F-test	**
Grand mean	2.91
CV	15.90%

Values represent mean $\pm$ standard error of fifteen replicates per treatment. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.01 probability level.

** Strongly significant at 0.01 probability level.

Table 4.3 Effects of MS medium supplemented with either 2,4-D with BAP and Kinetin or alone on the scores of callus induction from internodal explants of *M. macrocarpa* after 4 weeks of culture.

Composition (mg/l)	Mean score of callus
MS + 1.0 2,4-D + 1.0 BAP	1.60 ± 0.13^{c}
MS + 2.0 2,4-D + 1.0 BAP	3.00 ± 0.00^{d}
MS + 4.0 2,4-D + 1.0 BAP	0.47 ± 0.13^{a}
MS + 1.0 2,4-D + 1.0 KIN	1.60 ± 0.13^{c}
MS + 2.0 2,4-D + 1.0 KIN	1.33 ± 0.13^{bc}
MS + 4.0 2,4-D + 1.0 KIN	0.67 ± 0.13^{a}
MS + 0.5 2,4-D	1.80 ± 0.11^{c}
MS + 1.0 2,4-D	1.53 ± 0.13^{c}
MS + 1.5 2,4-D	1.60 ± 0.13^{c}
MS + 2.0 2,4-D	0.87 ± 0.09^{ab}
MS + 2.5 2,4-D	0.80 ± 0.11^{ab}
MS + 3.0 2,4-D	0.93 ± 0.01^{ab}
F-test	**
Grand mean	1.35
CV	32.54%

Values represent mean ± standard error of fifteen replicates per treatment. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.01 probability level.

** Strongly significant at 0.01 probability level.

4.2 Shoot multiplication

Ninety percent of *M. macrocarpa* seeds germinated within 10-14 days of inoculation on a growth regulator-free MS medium (data not shown). Nodal segments excised from 14-day-old axenic seedlings were used as explants in the present studies, and the morphogenic response of these explants to various BAP alone and NAA combined with BAP were documented. The addition of a cytokinin to the medium was essential to induce multiple shoots from the explants. The frequency of axillary shoot proliferation and the number of shoots per explant increased with increasing concentration of cytokinin up to an optimum level (Table 4.4, 4.6). BAP at a concentration of 2.0 mg/l induced multiple shoot buds in virtually all explants within 28 days (data not shown). The buds appeared as small green protuberances in the nodal explants that elongated into slender shoots possessing diminutive leaves (Figure 4.11). The highest number of shoots per explant was recorded to be 2.33 ± 0.13, on which formed in the MS medium supplemented with 0.5 and 2.0 mg/l BAP alone. They had an average length of 4.30 cm after 30 days. Multiple shoots were also induced in the nodal explants on MS and WPM medium containing NAA and BAP within 30 days (Figure 4.14). The optimum concentrations of NAA and BAP at which a maximum number of explants exhibited shoot development were 1.0 mg/l NAA and 1.5 mg/l BAP. However, the frequency of shoot proliferation in the media supplemented with BAP alone was relatively low and there were fewer shoots per explant compare with the media supplemented with NAA and BAP. BAP induced axillary shoot proliferation have been reported in several plant species such as *Phaseolus* species (Mallik and Saxena, 1992), *Arachis hypogaea* L. (Saxena *et al.*, 1992), *Wrightia tinctoria* R.Br. (Purohit and Kunda 1994). Moreover, the shoots that developed in this study failed to elongate (data not shown). Shoots that developed on multiplication media were transferred to modified MS media for rooting. Among various media used, MS medium supplemented with NAA 1.0 mg/l combined with BAP 1.5 mg/l showed excellent response. The nodal explants were inoculated on this MS medium responded maximum shoot multiplication (3.80 ± 0.11) per explant (Table 4.7) (Figure 4.6). Notwithstanding, the number of shoots that were achieved on MS medium supplemented with optimal concentrations of NAA 0.5 or 0.1 mg/l combined with concentration of BAP 1.0 or 1.5 mg/l were approximated to the first one (Table 4.6).

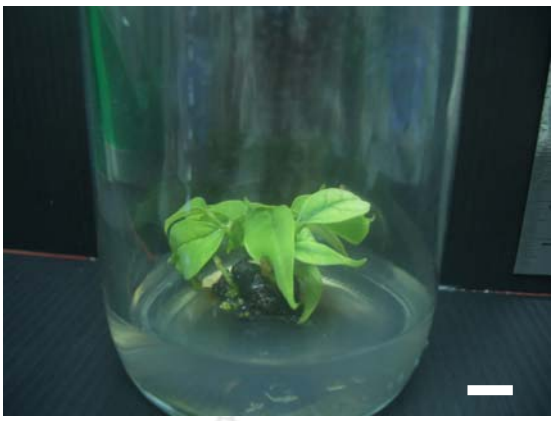

Figure 4.6 Nodal explants with multiple shoot development on MS medium supplemented with NAA 1.0 mg/l combined with BAP 1.5 mg/l showed maximum shoot multiplication per 3.80 $\pm$ 0.11explant after 4 weeks of the culture (Bar = 1 cm).

The mean values of shoot multiplication that were achieved on MS medium supplemented with plant growth regulators were higher than WPM (Table 4.6, 4.7). Increasing the concentration of BAP resulted in an increase in the rate of shoot regeneration ability. This is in contrast with the result reported in *Pterocarpus marsupium* (Anis et al., 2005) where higher concentrations of BAP resulted in a decrease in the rate of shoot regeneration ability.

Multiple shoots obtained were divided into clumps for further multiplication and to increase the number of shoots. Regular subculturing was done every 4 weeks onto fresh medium. During initial subculturing, the mother explant was kept intact with multiplied up shoots (Figure 4.7).

Figure 4.7 The mother explants were kept intact with multiplied up shoots *in vitro* during of the culture (Bar = 1 cm).

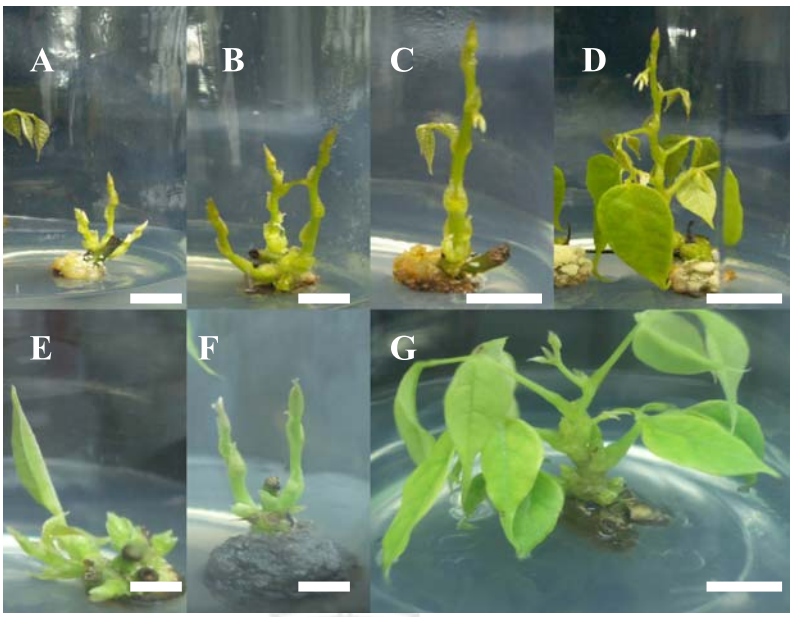

Figure 4.8 Nodal explants with multiple shoot development on MS medium supplemented with various concentration of NAA combined with BAP and BAP alone after 4 weeks of culture. (A), (B) Multiple shoots on MS medium supplemented with 0.5 mg/l BAP and 2.0 mg/l BAP, respectively (C), (D) The highest of shoot length was recorded to be 2.43 $\pm$ 0.04 cm on MS medium supplemented with 0.1 mg/l NAA and 1.5 mg/l BAP (E) Multiple shoots on MS medium supplemented with 0.5 mg/l NAA and 1.5 mg/l BAP (F), (G) Multiple shoots on MS medium supplemented with 1.5 BAP alone 2.27 $\pm$ 0.19 shoots per explant (Bar = 1 cm).

Table 4.4 Caulogenic response of nodal explants of *Mucuna macrocarpa* Wall. on MS supplemented with difference concentrations of BAP, after 4 weeks of culture.

Composition (mg/l)	Mean number of shoot
MS + 0 BAP	1.00 ± 0.00^{a}
MS + 0.5 BAP	2.33 ± 0.13^{b}
MS + 1.0 BAP	2.13 ± 0.17^{b}
MS + 1.5 BAP	2.27 ± 0.19^{b}
MS + 2.0 BAP	2.33 ± 0.13^{b}
F-test	**
Grand mean	2.01
CV	23.28%

Values represent mean $\pm$ standard error of fifteen replicates per treatment. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.01 probability level.

** Strongly significant at 0.01 probability level.

Table 4.5 Percentages of shoot multiplication form nodal explants of *Mucuna macrocarpa* Wall. after 4 weeks of culture on MS and WPM supplemented with NAA and BAP.

NAA (mg/l)	BAP (mg/l)	MS medium (%)	WPM (%)
0	0	0	0
0.1	0.5	100.0	100.0
0.1	1.0	100.0	86.7
0.1	1.5	100.0	86.7
0.5	0.5	93.3	66.7
0.5	1.0	100.0	80.0
0.5	1.5	100.0	73.3
1.0	0.5	86.7	80.0
1.0	1.0	80.0	80.0
1.0	1.5	100.0	100.0

Table 4.6 Effects of MS medium supplemented with NAA and BAP on number of shoot multiplication from nodal segment of *M. macrocarpa* on after 4 weeks of culture.

Composition (mg/l)	Mean number of shoot
MS + 0 NAA + 0 BAP	1.00 ± 0.00^{a}
MS + 0.1 NAA + 0.5 BAP	2.20 ± 0.24^{bc}
MS + 0.1 NAA + 1.0 BAP	3.27 ± 0.12^{c}
MS + 0.1 NAA + 1.5 BAP	3.33 ± 0.13^{c}
MS + 0.5 NAA + 0.5 BAP	1.73 ± 0.18^{b}
MS + 0.5 NAA + 1.0 BAP	3.00 ± 0.14^{c}
MS + 0.5 NAA + 1.5 BAP	3.40 ± 0.13^{c}
MS + 1.0 NAA + 0.5 BAP	2.07 ± 0.15^{bc}
MS + 1.0 NAA + 1.0 BAP	2.00 ± 0.17^{bc}
MS + 1.0 NAA + 1.5 BAP	3.80 ± 0.11^{d}
F-test	**
Grand mean	2.30
CV	25.87%

Values represent mean $\pm$ standard error of fifteen replicates per treatment. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.01 probability level.

** Strongly significant at 0.01 probability level.

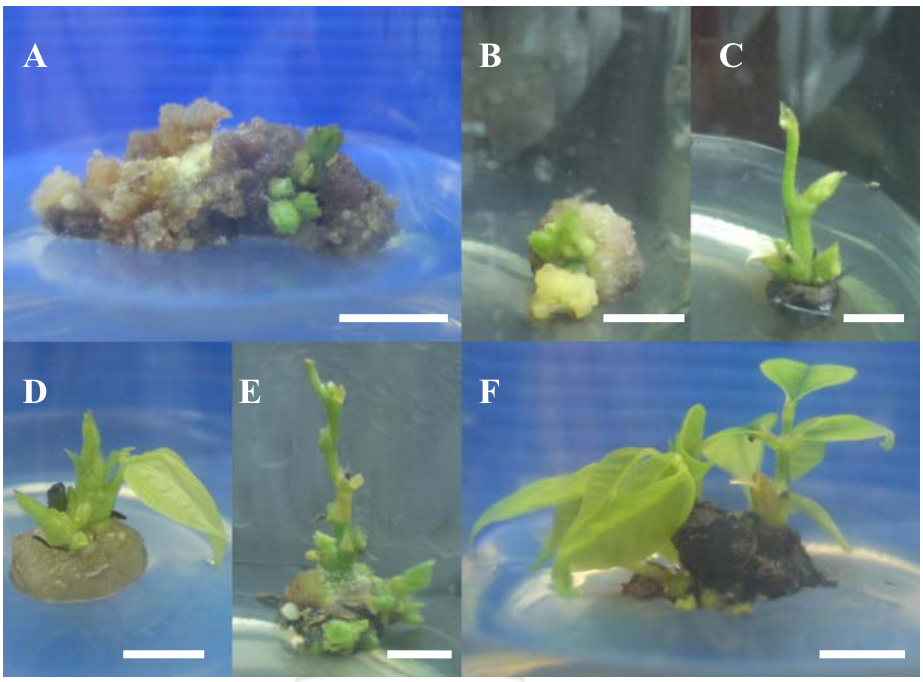

Figure 4.9 Effects of NAA and BAP on the adventitious bud initiation of divided nodal explants. Four weeks after inoculation on MS and WPM medium (Bar = 1 cm).

Table 4.7 Effects of NAA and BAP on number of shoot multiplication from nodal segment of *M. macrocarpa* on MS medium after 4 weeks of culture.

Composition (mg/l)	Mean number of shoot
WPM + 0 NAA + 0 BAP	1.00 ± 0.00^{a}
WPM + 0.1 NAA + 0.5 BAP	2.00 ± 0.22^{bc}
WPM + 0.1 NAA + 1.0 BAP	1.80 ± 0.15^{c}
WPM + 0.1 NAA + 1.5 BAP	1.67 ± 0.13^{c}
WPM + 0.5 NAA + 0.5 BAP	1.93 ± 0.21^{b}
WPM + 0.5 NAA + 1.0 BAP	2.00 ± 0.17^{c}
WPM + 0.5 NAA + 1.5 BAP	1.80 ± 0.15^{c}
WPM + 1.0 NAA + 0.5 BAP	2.33 ± 0.13^{bc}
WPM + 1.0 NAA + 1.0 BAP	2.27 ± 0.21^{bc}
WPM + 1.0 NAA + 1.5 BAP	3.33 ± 0.13^{d}
F-test	**
Grand mean	2.30
CV	25.87%

Values represent mean $\pm$ standard error of fifteen replicates per treatment. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.01 probability level.

** Strongly significant at 0.01 probability level.

Table 4.8 Effects of NAA and BAP on the length of shoot derived from nodal segment of *M. macrocarpa* on MS medium, after 4 weeks of culture.

Composition (mg/l)	Mean of shoot length
MS + 0 NAA + 0 BAP	0.94 ± 0.03^{a}
MS + 0.1 NAA + 0.5 BAP	1.41 ± 0.04^{b}
MS + 0.1 NAA + 1.0 BAP	1.62 ± 0.06^{c}
MS + 0.1 NAA + 1.5 BAP	2.43 ± 0.04^{f}
MS + 0.5 NAA + 0.5 BAP	1.39 ± 0.04^{b}
MS + 0.5 NAA + 1.0 BAP	1.51 ± 0.04^{bc}
MS + 0.5 NAA + 1.5 BAP	2.13 ± 0.07^{e}
MS + 1.0 NAA + 0.5 BAP	1.36 ± 0.04^{b}
MS + 1.0 NAA + 1.0 BAP	1.51 ± 0.05^{bc}
MS + 1.0 NAA + 1.5 BAP	1.89 ± 0.06^{d}
F-test	**
Grand mean	1.54
CV	12.53%

Values represent mean $\pm$ standard error of fifteen replicates per treatment. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.01 probability level.

** Strongly significant at 0.01 probability level.

Table 4.9 Effects of WPM medium supplemented with NAA and BAP on the length of shoot derived from nodal segment of *M. macrocarpa,* after 4 weeks of culture.

Composition (mg/l)	Mean of shoot length (cm)
WPM + 0 NAA + 0 BAP	0.84 ± 0.03^{a}
WPM + 0.1 NAA + 0.5 BAP	1.31 ± 0.03^{b}
WPM + 0.1 NAA + 1.0 BAP	1.55 ± 0.05^{c}
WPM + 0.1 NAA + 1.5 BAP	1.95 ± 0.07^{f}
WPM + 0.5 NAA + 0.5 BAP	1.24 ± 0.03^{b}
WPM + 0.5 NAA + 1.0 BAP	1.44 ± 0.04^{bc}
WPM + 0.5 NAA + 1.5 BAP	1.81 ± 0.05^{e}
WPM + 1.0 NAA + 0.5 BAP	1.28 ± 0.04^{b}
WPM + 1.0 NAA + 1.0 BAP	1.40 ± 0.08^{bc}
WPM + 1.0 NAA + 1.5 BAP	1.73 ± 0.03^{d}
F-test	**
Grand mean	1.54
CV	12.53%

Values represent mean $\pm$ standard error of fifteen replicates per treatment. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.01 probability level.

** Strongly significant at 0.01 probability level.

4.3 Root induction

MS medium without any growth regulators failed to induce root formation in the regenerated shoots even after 30 days. Of the two auxin supplements used to induce root formation NAA was most effective. Both MS medium containing NAA and IBA alone induced rooting in 100% of the shoots within 18–20 days (data not shown); in 30 days, about two roots had formed per shoot (Table 4.15), and roots averaged 2.46 cm in length. The highest number of rooting was markedly enhanced by supplementing the medium with a combination of 2.0 mg/l NAA(Table4.15) (Figure 4.10). The roots were prominent with several lateral roots.

The presence of these two auxins favoured root induction in several tree species, such as *Cinnamomum zeylanicum* Breyn. (RavishankarRai & Jagadishchandra, 1987) and *Morus* species (Pattnaik & Chand 1997). The rooted plantlets were successfully acclimatized for 4 weeks followed by another 2 weeks in the shade and eventually established outdoors under full sun (Figure 4.22). Eighty percent of the plants transferred to soil survived (data not shown). The regenerated plants did not show any detectable phenotypic variation.

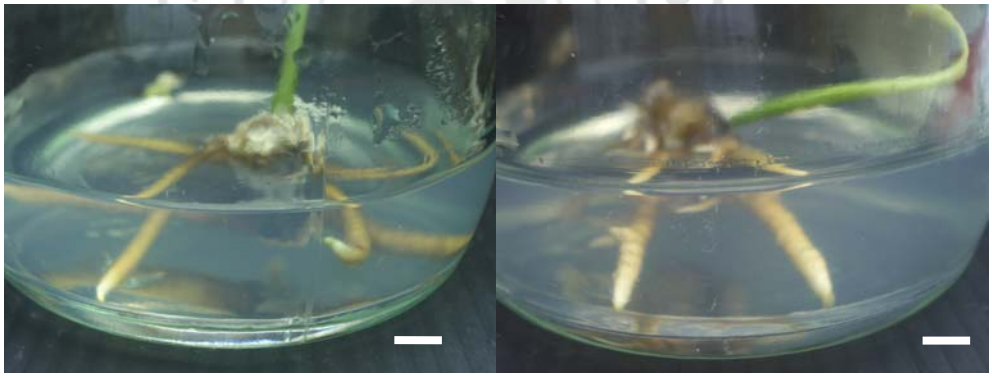

Figure 4.10 Development of roots from the base of *in vitro* regenerated shoot of *M. macrocarpa* on MS supplemented with 2.0 mg/l NAA after 4 weeks of culture (Bar 1 cm).

Table 4.10 Effects of MS medium supplemented with NAA and IBA on length of root induction from shootlets of *M. macrocarpa* after 4 weeks of culture.

Composition (mg/l)	Mean root length (cm)
MS + 0 NAA	0.00 ± 0.00^{a}
MS + 0.5 NAA	1.30 ± 0.05^{b}
MS + 1.0 NAA	2.63 ± 0.06^{c}
MS + 1.5 NAA	3.90 ± 0.24^{d}
MS + 2.0 NAA	4.73 ± 0.07^{e}
MS + 0.5 IBA	1.16 ± 0.07^{b}
MS + 1.0 IBA	4.62 ± 0.07^{d}
MS + 1.5 IBA	2.26 ± 0.03^{c}
MS + 2.0 IBA	1.58 ± 0.04^{b}
F-test	**
Grand mean	2.46
CV	12.13%

Values represent mean $\pm$ standard error of fifteen replicates per treatment. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.01 probability level.

** Strongly significant at 0.01 probability level.

Table 4.11 Rhizogenic response on numbers of root of *in vitro* regenerated shoot of *M. macrocarpa* on MS medium supplemented with different concentrations of NAA and IBA alone, after 4 weeks of culture.

Composition (mg/l)	Mean number of root per shoot
MS + 0 NAA	0.00 ± 0.00^{a}
MS + 0.5 NAA	1.60 ± 0.16^{b}
MS + 1.0 NAA	2.60 ± 0.16^{d}
MS + 1.5 NAA	3.30 ± 0.21^{e}
MS + 2.0 NAA	4.30 ± 0.21^{f}
MS + 0.5 IBA	1.10 ± 0.10^{b}
MS + 1.0 IBA	1.90 ± 0.10^{c}
MS + 1.5 IBA	1.30 ± 0.15^{bc}
MS + 2.0 IBA	1.70 ± 0.15^{bc}
F-test	**
Grand mean	1.98
CV	24.48%

Values represent mean $\pm$ standard error of fifteen replicates per treatment. Mean values within the column followed by the same letter are not significantly different by the Tukey's test at 0.01 probability level.

** Strongly significant at 0.01 probability level.

Figure 4.11 One-month-old regenerated plants of *M. macrocarpa* in the greenhouse.

CHAPTER V

CONCLUSION

5.1 Callus induction

Creamy compact callus initiated on MS medium and WPM with 2.0 mg/l α-Naphthaleneacetic acid (NAA) and 1.0 mg/l 6-Benzyl-aminopurine (BAP). The highest growth and size increasing were observed in the callus cultured on MS medium containing 2.0 mg/l 2,4-Dichlorophenoxyacetic acid (2,4-D) and 1.0 mg/l BAP.

5.2 Shoot multiplication

This part of the research emphasizes microcuttings obtained from shoot multiplication because this is by far the most utilized methodology and where adventitious rooting has occurred. The optimum concentrations of NAA and BAP at which a maximum number of explants exhibited shoot development were 1.0 mg/l NAA and 1.5 mg/l BAP. MS media is better than WPM.

5.3 Root induction

The stock plant in a tissue culture system is contained in a sterile, highly defined environment, the degree of control of factors predisposing the cutting to rooting is much greater than with the stock used for the more classical propagation. Thus the quality of *in vitro* produced cuttings can be managed to a high degree so that the formation of adventitious roots can be

obtimized. The highest number of rooting was markedly enhanced by supplementing the medium with a combination of 2.0 mg/l NAA. The rooted plantlets were successfully acclimatized for 4 weeks. The regenerated plants did not show any detectable phenotypic variation.

Figure 5.1 A stabilized shoot culture of *Mucuna macrocarpa* Wall. (left); and a micropropagules rooted and partially acclimated in vermiculite (right).

5.4 Acclimatization

Woody plants have not only been more difficult to establish in culture, but problems with the rooting and acclimatization of *in vitro* produced propagules have complicated removal from culture and subsequent commercialization.

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APPENDIX A

MEDIA COMPOSITION

Table 1 Composition of MS (Murashige and Skoog, 1962) medium

Component	mg/l
Major salts	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Minor salts	
KI	0.83
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSo_4.5H_2O$	0.025
$Na_2.EDTA$	37.3
$FeSO_4.7H_2O$	27.8
Vitamins and organics	
myo-Inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2.0
Sucrose	30 g
pH	5.8

Table 2 Composition of WPM (McCown and Lloyd, 1981) woody plant medium

Component	mg/l
Macronutrients	
NH_4NO_3	400
$Ca(NO_3)_2.4H_2O$	556
K_2SO_4	990
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$CaCl_2.2H_2O$	96
Micronutrients	
H_3BO_3	6.2
$MnSO_4.H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSo_4.5H_2O$	0.25
$FeSO_4.7H_2O$	27.8
Na_2.EDTA	37.3
Vitamins and organics	
Thiamine HCl	1.6
Nicotinic acid	0.5
myo-Inositol	100
Sucrose	20 g
pH	5.6

APPENDIX B

STOCK SOLUTION

Table 3 MS (Murashige and Skoog, 1962) medium stock solution

	Chemical constituents	Concentration (g/l)
Stock I (50x)	MS-major salts	
(use 20 ml/l)	KNO_3	95
	NH_4NO_3	82.5
	$CaCl_2.2H_2O$	22
Stock II (100x)	MS-minor salts	
(use 10 ml/l)	H_3BO_3	0.62
	KH_2PO	17
	KI	0.083
	$Na_2MoO_4.2H_2O$	0.025
	$CoCl_2.6H_2O$	0.0025
Stock III (100x)	Sulfate Stock	
(use 10 ml/l)	$MgSO_4.7H_2O$	37
	$MnSO_4.H_2O$	1.69
	$ZnSO_4.7H_2O$	0.86
	$CuSO_4.5H_2O$	0.0025
Stock IV (100x)	EDTA sodium ferric salt	
(use 10 ml/l)	$Na_2EDTA.2H_2O$	3.725
	$FeSO_4.7H_2O$	2.785
Stock V (100x)	MS-vitamins	
(use 10 ml/l)	Glycine	0.2
	Nicotinic acid	0.05
	Thiamine-HCl (B1)	0.01
	Pyridoxine-HCl (B6)	0.05
	myo-Inositol	10

Table 4 WPM (McCown and Lloyd, 1981) woody plant medium stock solution

	Chemical constituents	Concentration (g/l)
Stock I (50x)		
(use 20 ml/l)	NH_4NO_3	20
	$Ca(NO_3)_2.4H_2O$	27.8
Stock II (50x)		
(use 20 ml/l)	K_2SO_4	49.5
Stock III (200x)		
(use 5 ml/l)	KH_2PO_4	34
	H_3BO_3	1.24
	$Na_2MoO_4.2H_2O$	0.05
Stock IV (200x)		
(use 5 ml/l)	$CaCl_2.2H_2O$	9.2
Stock V (200x)		
(use 5 ml/l)	$MgSO_4.7H_2O$	74
	$MnSO_4.H_2O$	7.46
	$ZnSO_4.7H_2O$	1.72
	$CuSO_4.5H_2O$	0.05
Stock VI (200x)		
(use 5 ml/l)	EDTA	7.45
	$FeSO_4.7H_2O$	5.57
Stock VII (200x)		
(use 5 ml/l)	Glycine	0.4
	Nicotinic acid	0.1
	Thiamine-HCl (B1)	0.2
	Pyridoxine-HCl (B6)	0.1
	myo-Inositol	20

Preparation of Murashige & Skoog (MS) stocks

The formulation of Murashige & Skoog's (1962) medium is given in Table 3. Additional formulations are given in Table 1.

MS-major salts stock (50x; Table 3, Stock I). Add approximately 400 ml Reverse Osmosis (R.O.) water to a 1-litre beaker. Weigh and dissolve each of the salts given in the third column using a magnetic stirrer. Transfer the solution to a 1-litre volumetric flask, and add R.O. water to the final volume. Store under refrigeration. Pipette 20 ml of the major salts stock for 1 liter of MS nutrient medium.

MS-minor salts stock (100x; Table 3, Stock II). Add approximately 400 ml R.O. water to a 1-litre beaker. Weigh and dissolve each of the salts given in the third column using a magnetic stirrer. Transfer the solution to a 1-litre volumetric flask, and add R.O. water to the final volume. Store under refrigeration. Pipette 10 ml of the minor salts stock for 1 liter of MS nutrient medium.

MS-sulfate stock (100x; Table 3, Stock III). Add approximately 400 ml R.O. water to a 1-litre beaker. Weigh and dissolve each of the salts given in the third column using a magnetic stirrer. Transfer the solution to a 1-litre volumetric flask, and add R.O. water to the final volume. Store under refrigeration. Pipette 10 ml of the sulfate stock for 1 liter of MS nutrient medium.

MS-EDTA sodium ferric salt stock (100x; Table 3, Stock IV). Dissolve $FeSO_4.7H_2O$ in 40 ml of warm R.O. water in a 100-cm^3 beaker. In a separate beaker dissolve $Na_2EDTA.2H_2O$ in 40 ml of warm R.O. water. Mix the two solutions and transfer to a 1-litre volumetric flask. Add R.O. water to the final volume. The iron stock should be protected from light by storing the solution in an amber bottle, or wrap the entire flask with aluminum foil. Store under refrigeration. Pipette 10 ml of the EDTA sodium ferric salt stock for 1 liter of MS nutrient medium.

MS-vitamins stock(100x; Table 3, Stock V). Add about 400 ml R.O. water to a 1-litre beaker. Weigh and dissolve each of the salts given in the third column using a magnetic stirrer. Transfer the solution to a 1-litre volumetric flask, and add R.O. water to the

final volume. Store under refrigeration. Pipette 10 ml of the sulfate stock for 1 liter of MS nutrient medium.

Note: Do not pipette directly from stock bottles, and do not return any unused stock solutions to the stock bottles. Label all stock solutions and include the concentration, your initials, and the date of preparation. Although inorganic salts are relatively stable in solution under refrigeration, vitamin stock should be discarded after 30 days. Also, vitamin stock should be visually examined periodically for any signs of microorganisms.

Preparation of the complete MS medium

1. Add approximately 400 ml R.O. water to a 1-litre beaker.
2. Pipette each of the MS stock solutions: 20 ml stock I, 10 ml stock II, 10 ml stock III, 10 ml stock IV, 10 ml stock vitamins. Mix it using a magnetic stirrer..
3. Weigh 30 g sucrose and dissolve it in the medium mixture.
4. Add R.O. water until the total volume of liquid is about 800 cm^3. While agitating the solution with a magnetic stirrer, adjust the pH to 5.8 with droplets of 1N NaOH or 1N HCl with separate Pasteur pipettes.
5. Transfer the medium to a 1-litre volumetric flask and add R.O. water to the final volume.
6. Weigh 0.245 g Phytagel™ and dissolve it in the medium mixture using a magnetic stirrer. Cover beaker with clear film protecting volatilization.
7. Boil in a microwave for 10 minutes. Each of wide necked bottle with fitted lids sized 8 Onz. can be poured with 30 ml medium and then autoclave at 121 $^{\circ}C$ for 20 min.
8. After the sterilized medium is removed from the autoclave, the bottles are swirled for a few minutes to ensure the dissolution of the medium. After the gel in the bottles has cooled, store in clean cabinet.

Note: WPM is also prepared with the same steps.

APPENDIX C

GLOSSARY

Acuminate tapering gradually to a point

Adventitious Developing from unusual points of origin, such as shoot or root tissues, from callus or embryos, from sources other than zygotes.

Agar a polysaccharide powder derived from algae used to gel a medium. Agar is generally used at a concentration of 6-12 g/liter.

Aseptic Free of microorganisms.

Aseptic Technique Procedures used to prevent the introduction of fungi, bacteria, viruses, mycoplasma or other microorganisms into cultures.

Autoclave A machine capable of sterilizing wet or dry items with steam under pressure. Pressure cookers are a type of autoclave.

Auxin A group of plant growth regulators that promotes callus growth, cell division, cell enlargement, adventitious buds, and lateral rooting. Endogenous auxins are auxins that occur naturally. Indole-3-acetic (IAA) is a naturally occurring auxin. Exogenous auxins are auxins that are man-made or synthetic. Examples of exogenous auxins included 2,4-Dichlorophenoxyacetic acid (2,4-D), Indole-3-Butyric acid (IBA),α-Naphthaleneacetic acid (NAA), and 4-Chlorophenoxyacetic acid (CPA).

Auxins extend cells and are important root growth regulators in cultures. Starts cell division (mitosis) in connection to cytokinins. Auxins stimulate cells to undergo embryogenesis; at higher concentrations there is a callus proliferation and at lower concentrations, the rooting effect dominates. These substances counteract axillary growth and bud formation. With gibberellines, auxin promote fruit set.

2,4-D 2,4-dichlorophenoxyethanoic acid. A herbicide used mostly fight growth of certain dicotyledons (not efficient against monocotyledons). In cultures it's added to keep up a callus cell

proliferation. It also suppress the differentiation (organogenesis) heavily. (Similar to 2, 4, 5-T, or 'Orange', which was used in Vietnam during the war.)

NAA α-naphtalene acetic acid. Stimulates rooting at lower concentrations (0.5-1.0 mg/l) and callus proliferation at higher concentrations; exogenic (analogous to IAA).

IAA Indole-3-ethanoic acid. Controls the organogenesis of shoot cells and cell division in roots naturally in plants; endogenic. Can start callus proliferation in cultures (at higher concentrations).

IBA Indole-3-buturic acid. Used to induce rooting of shoots (e.g. of root-free shoots derived from a culture).

PAA phenylethanoic acid. Extends cells and may induce a production of shoots together with cytokinins.

Callus An unorganized, proliferate mass of differentiated plant cells, a wound response.

Clonal Propagation Asexual reproduction of plants that are considered to be genetically uniform and originated from a single individual or explant.

Contamination Being infested with unwanted microorganisms such as bacteria or fungi.

Culture A plant growing *in vitro*.

Cytokinin A group of plant growth regulators that regulate growth and morphogenesis and stimulate cell division. Endogenous cytokinins, cytokinins that occur naturally, include zeatin and 6-3,3-dimethylallylaminopurine (2iP). Exogenous cytokinins, cytokinins that are man-made or synthetic, include 6-furfurylaminop-urine (kinetin) and 6-benzylaminopurine (BA or BAP).

Cytokinins have the effect of increasing the mitosis (cell division) of shoot and buds. Some cytokinins increase the size of the plant cells and e.g. makes leaves larger. The cell differentiation can be controlled (e.g. for production of a large amount of calli-cells).

Growth of roots is inhibited. Cytokinins are adenine derivatives and kinetin can e.g. be synthesised from heating nucleic acid. Coconut water (the liquid interior of a coconut) contains cytokinins to stimulate the growth of the coconut palm.

Kinetin 6-furfurylaminopurine. Exogenic but almost identical to natural substances. Often used in cultures. Stimulates shoot and leaf growth at lower concentrations (callus proliferation at higher concentrations).

BA N^6-benzyladenine. Synthetic cytokinin just like kinetin.

BAP 6-benzylaminopurine. Stimulates a production of adventitious shoot and callus; see description of kinetin.

Zeatin Naturally occuring cytokinin (first isolated from corn).

Differentiated Cells that maintain, in culture, all or much of the specialized structure and function typical of the cell type in vivo. Modifications of new cells to form tissues or organs with a specific function.

Explant Tissue taken from its original site and transferred to an artificial medium for growth or maintenance.

Gibberellins A plant growth regulator that influences cell enlargement. Endogenous growth forms of gibberellin include Gibberellic Acid (GA_3), Gibberellins and other substances:

Cells are extended (there's especially an extension of internodes) and cells get larger from gibberellins. The flower production is probably stimulated and the metabolism of many seeds are regulated by gibberellins (e.g. mobilization of energy storing substances in seeds and a stimulation of amylase production to digest starches); meaning that these substances may 'wake' certain seed up from resting stages. Seed-less grapes are grown while treated with gibberellines.

GA Gibberellinic acid. A treatment of seeds may stimulate germination. The growth of shoot is also stimulated, while root formation is inhibited.

Ethylene CH_2C_2 Ethylene is produced by ripening and even decaying plants. There is a reason to why one shouldn't place certain plants close to a fruit bowl.

Horizontal laminar flow unit An enclosed work area that has sterile air moving across it. The air moves with uniform velocity along parallel flow lines. Room air is pulled into the unit and forced through a HEPA (High Energy Particulate Air) filter, which removes particles 0.3 μm and larger.

Hormones Growth regulators, generally synthetic in occurrence, that strongly affects growth (i.e. cytokinins, auxins, and gibberellins).

Internode The space between two nodes on a stem

In vitro To be grown in glass (Latin). Propagation of plants in a controlled, artificial environment using plastic or glass culture vessels, aseptic techniques, and a defined growing medium.

In vivo To be grown naturally (Latin)

Media Plural of medium

Medium A nutritive solution, solid or liquid, for culturing cells.

Microculture *In vitro* cultures; suggested as a replacement for the term “tissue culture.”

Microcutting A shoot derived from microculture, usually shoot culture, that is to be rooted.

Macrocutting Conventional cutting taken from stock plants grown *ex vitro.*

Micropropagation *In vitro* Clonal propagation of plants from shoot tips or nodal explants, usually with an accelerated proliferation of shoots during subcultures.

Micropropagule (microplant) A plant derived from microculture, also termed plantlet.

Node A part of the plant stem from which a leaf, shoot or flower originates.

Passage The transfer or transplantation of cells or tissues with or without dilution or division, form one culture vessel to another.

Passage Number The number of times the cells or tissues in culture have been subcultured or passaged.

Pathogen A disease-causing organism.

Pathogenic Capable of causing a disease.

Petiole A leaf stalk; the portion of the plant that attaches the leaf blade to the node of the stem.

Plant Tissue Culture The growth or maintenance of plant cells, tissues, organs or whole plants *in vitro*.

Regeneration In plant cultures, a morphogenetic response to a stimulus that results in the products of organs, embryos, or whole plants.

Shoot apical meristem Undifferentiated tissue, located within the shoot tip, generally appearing as a shiny dome-like structure, distal to the youngest leaf primordium and measuring less that 0.1 mm in length when excised.

Shoot culture Multiplication based principally on the stimulation of axillary buds of shoots growth in microculture, although adventitious shoot meristem development may also be involved.

Somaclonal Variation Phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones.

Somaclones Plants derived from any form of cell culture involving the use of somatic plant cells.

Stage I A step in *in vitro* propagation characterized by the establishment of an aseptic tissue culture of a plant.

Stage II A step in *in vitro* propagation characterized by the rapid numerical increase of organs or other structures.

Stage III A step in *in vitro* propagation characterized by preparation of propagules for successful transfer to soil, a process involving rooting of shoot cuttings, hardening of plants, and initiating the change from the heterotrophic to the autotropic state.

Stage IV A step in *in vitro* plant propagation characterized by the establishment in soil of a tissue culture derived plant, either after undergoing a Stage III pretransplant treatment, or in certain species, after the direct transfer of plants from Stage II into soil.

Sterile (A) Without life. (B) Inability of an organism to produce functional gametes. (C) A culture that is free of viable microorganisms.

Sterile Techniques The practice of working with cultures in an environment free from microorganisms.

Subculture See "Passage". With plant cultures, this is the process by which the tissue or explant is first subdivide, then transferred into fresh culture medium.

Tissue culture Refers to *in vitro* culture in general. More properly, tissue culture is the growth in vitro of organized tissue systems such as the epidermis or vascular elements.

Totipotency A cell characteristic in which the potential for forming all the cell types in the adult organism are retained.

Undifferentiated With plant cells, existing in a state of cell development characterized by isodiametric cell shape, very little or no vacuole, a large nucleus, and exemplified by cells comprising an apical meristem or embryo.

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