UNIVERSITI PUTRA MALAYSIA

HISTOLOGICAL ANALYSES AND DETECTION OF AZADIRACHTIN FROM IN VITRO CULTURES OF SENTANG (AZADIRACHTA EXCELSA (JACK) JACOBS)

YOHANNES ADANE MIHIRET.

FBSB 2005 29

Dedicated with love to:

My Father, Kibur Ato Adane Mihiret Ferede

and

My Mother, Kibirt W/ro Asegedech Seyoum Biratu

Abstract of thesis Presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Masters of Science

HISTOLOGICAL ANALYSES AND DETECTION OF AZADIRACHTIN FROM *IN VITRO* CULTURES OF SENTANG (*AZADIRACHTA EXCELSA* [JACK] JACOBS)

By

YOHANNES ADANE MIHIRET

June 2005

Chairman: **Associate Professor Norihan Mohd Saleh, PhD**

Faculty: **Biotechnology and Biomolecular Sciences**

The growth profiles of calli and suspension cultures from leaf segments of *in vitro* grown *Azadirachta excelsa* shoots were studied to establish plant regeneration system and to quantitatively detect the presence of azadirachtin in suspension cultures. A highly significant variation ($p < 0.01$) was observed among the plant growth regulators used in the callogenesis experiments. Explants treated with callus induction media (IM) containing MS medium, Murashige and Skoog's (1962), supplemented with 9 µM 2,4-Dichlorophenoxy-acetic acid (2,4-D) and 4.4 µM Benzyl Amino Purine (BAP) showed the highest callusing efficiency (94%). But, calli with meristematic features (semi-friable to friable texture and pale yellow to yellow color) were obtained from 88% of explants on IM containing MS supplemented with 36.2 µM 2,4-D.

Three morphologically different types of calli could be observed on proliferation media (PM); i.e. friable and pale yellow calli, sometimes with nodules and watery appearance

(Type-1); semi friable, nodular, proliferative and pale yellow to white calli (Type-2); and semi-compact calli with heterogeneous appearance (pale beige (brown) at the base with pale and granular areas at the surface) (Type-3). Maximum fresh weight of calli with meristematic features (8.6 g) was obtained using PM containing 2.26 µM 2,4-D and 33.3 µM BAP. Growth rate studies on these calli indicated that a 2-3 weeks subculturing cycle was most suitable.

In cell suspension cultures, maximum cell-biomass increase (1.36 $\pm$ 0.041 g) was obtained in media containing 2.26 µM 2,4-D and 0.22 µM thidiazuron (TDZ) after six weeks of culture incubation (with a weekly subculturing cycle). Nevertheless, growth rate studies indicated that 12-15 days was the end of the exponential growth phase (maximum average settled cell volume (4.5 mL) was attained). Thus, a 12-15 days subculturing cycle is recommended. Cells released into the various suspension media formed fine cell aggregates which further developed to globular like structures, which, upon transfer to growth regulator free media, produced unipolar shoot primordia (visible to the naked eye), with a 63% conversion rate.

Histological studies at various stages of calli development clearly showed progressive structural changes of cells (in calli) into meristematic groupings (aggregates), and hence shoot primordia. The study showed marked morphological differences among the different calli. Type-1 calli had smaller sized cells appearing in clusters and with prominent nucleus. Type-2 calli had isodiametric, but randomly dispersed groups of dividing cells. Type-3 calli had larger and highly vacuolated internal cells and actively

dividing external cells. Cell aggregates also had low to moderate intercellular polysaccharide contents coupled with asymmetric cell division patterns. Moreover, regenerants from the cell aggregates showed sufficient precambial attachment to the parent tissue suggesting organogenesis (instead of embryogenesis) as regeneration pathway.

Azadirachtin was detected and quantified in different cell aggregate samples from suspension cultures, using high performance liquid chromatography (HPLC). Samples varied significantly with regard to the accumulation of azadirachtin. Moreover, the accumulation of the substance in the samples was dependent on the type of growth regulator used. Hence, 2,4-D (2,4-Di-chlorophenoxy-acetic acid) treated samples showed the highest (0.966 mg/g) azadirachtin content, as compared to NAA (Naphthalene Acetic Acid) treated samples (0.793 mg/g).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

HISTOLOGICAL ANALYSES AND DETECTION OF AZADIRACHTIN FROM *IN VITRO* CULTURES OF SENTANG (*AZADIRACHTA EXCELSA* [JACK] JACOBS)

Oleh

Yohannes Adane Mihiret

Jun 2005

Pengerusi: Profesor Madya Norihan Mohd Saleh, PhD

Fakulti : Bioteknologi dan Sains Biomolekul

Profil pertumbuhan kalus dan kultur ampaian daripada segmen daun *Azadiracht. excelsa* dikaji untuk membangunkan sistem regenerasi di samping mengenalpasti dan menganalisa jumlah azadirachtin dalam setiap sampel. Variasi yang amat signifikan ($p < 0.01$) telah diperolehi di antara hormon pengawalaturan dari eksperimen kalogenesis. Eksplan yang dirawat dengan media induksi kalus (IM) mengandungi medium MS yang ditambah dengan 9 µM 2,4-D dan 4.4 µM BAP menunjukkan efisiensi pembentukan kalus yang tertinggi (94%). Namun demikian, efisiensi tertinggi bagi kalus yang mempunyai ciri meristematik diperolehi daripada eksplan yang dirawat dengan IM bersama 36.2 µM 2,4-D (88.3 ±2.24%).

Terdapat tiga jenis morfologi kalus yang kelihatan pada media proliferasi (PM); iaitu: separa lerai berwarna kuning pucat, kadangkala bernodul dan berair (Jenis-1); separa lerai, bernodul berupaya untuk mengganda dan kalus berwarna kuning pucat ke putih (Jenis-2); separa tumpat dan bersifat heterogenus, berwarna coklat pucat di sebelah

bawah kalus manakala bergranul di bahagian permukaan (Jenis-3). Berat segar maksimum kalus yang berciri meristematik sebanyak (8.6 g) telah diperolehi dari PM yang mengandungi 2.26 μM 2,4-D dan 33.3 μM BAP. Kajian kinetik pertumbuhan kalus menunjukkan bahawa subkultur sesuai dilakukan setiap 2-3 minggu selepas inkubasi kultur.

Peningkatan sel biomas (1.36 ± 0.041 g) bagi kultur sel ampaian diperolehi dari media MS yang mengandungi 2.26 μM 2,4-D dan 0.22 μM TDZ selepas diinkubasi selama 6 minggu (dengan pusingan subkultur pada setiap minggu). Oleh sebab itu, kajian kinetik pertumbuhan menunjukkan 12-15 hari merupakan fasa pertumbuhan eksponen yang maksimum (purata maksimum isipadu sel terampai (4.5 mL) diperolehi). Oleh itu, subkultur 12-15 hari selepas diinkubasi adalah disyorkan. Sementara itu, sel yang dibebaskan didalam sel ampaian akan membentuk aggeregat berbentuk struktur globular. Sekiranya dipindahkan ke media tanpa hormon, struktur ini menghasilkan primordia pucuk bersifat unipolar (dapat dilihat dengan mata kasar) dengan kadar penghasilan 63%.

Kajian histologi pada pelbagai peringkat perkembangan kalus menunjukkan perubahan struktur sel (di dalam kalus) secara progresif kepada kumpulan meristematik (aggeregat), dan seterusnya ke primordia pucuk . Kajian ini menunjukkan perbezaan morfologi di antara kalus. Kalus Jenis-1 mengandungi saiz sel yang kecil dan berkelompok dan setiap sel mengandungi nukleus yang jelas. Kalus Jenis-2 mengandungi sel isodiametrik dan kumpulan sel yang aktif membahagi. Bagi kalus

Jenis-3, sel dalaman mempunyai vakuol yang besar dan banyak, manakala sel luaran adalah giat membahagi. Sel aggregat mengandungi kandungan polisakarida interselular yang rendah dan sederhana serta menunjukkan corak pembahagian sel yang tidak simetri. Selain itu, regenerasi daripada aggeregat menunjukkan percantuman prokambial dengan tisu induk, ini mencadangkan regenerasi berlaku melalui proses organogenesis (bukan embryogenesis).

Kehadiran azadirachtin telah dikesan dan dikenalpasti pada sampel sel aggeregat daripada kultur ampaian yang berlainan, dengan menggunakan kromatografi cecair berprestasi tinggi (HPLC). Lagipun, penghimpunan azadirachtin di dalam sampel adalah bergantung pada jenis hormon pengawalaturan yang digunakan. Sampel-sampel ampaian sel menunjukkan perbezaan ketara dari segi pengumpulan azadirachtin. Sampel yang dirawat dengan 2,4-D mengandungi kepekatan azadirachtin tertinggi (0.966 mg/g), berbanding dengan sampel yang dirawat dengan NAA (0.793 mg/g).

ACKNOWLEDGEMENTS

ወስባሐት ለእግዚአብሔር ! Glory be to God, for He has given me the strength to shoulder all the rigors of the postgraduate study I have just completed. Praise be to His great and awesome name, for He has bowed His ears to my supplications. I extol God, the most gracious and kind, for He has made me a sheep in His pasture.

My special thankfulness is due to my supervisor, Associate Professor Dr. Norihan Mohd Saleh. This achievement would have been unattainable without her guidance, mentoring and constant supervision. She showed never-ending patience for this work; and above all, she has led me to the fascinating world of plant tissue culture. My appreciations and thankfulness are also to Dr. Kodi Isparan Kandasamy, who was the key member of the supervisory committee, from its centennial. His support, supervision and invaluable comments have always been my motivations. Associate Professor Dr. Abdul Ghani Yunus and Professor Dr. Yazid Mohd Manap were also key members of the supervisory committee with priceless technical supports and encouragements.

My heartfelt gratitude goes to my wonderful parents, my father Ato Adane Mihiret and my mother W/ro Asegedech Seyoum. They have given me all the best they could to help me be the person I am. This piece of work is dedicated to them without any reservation. I also want to thank my sisters and brothers for their love and support throughout my studies and in everyday life. My special gratefulness is also to my fiancée and future wife, Lidya Tekeste Weresom. Her constant prayers, encouragement and unconditional love have inspired me in so many ways.

Many thanks are also to my labmates for the mutual respects and companionship we shared through out those weary days in the lab. Special thank you is due to Noraliza Binti Alias, for her help and orientations during the early days of the research project. I also want to thank the staffs at the tissue culture laboratories of the department of Cell and Molecular Biology (UPM), the tissue culture laboratory of the Forestry Research Institute of Malaysia (FRIM), and the histology laboratory of Crop Science Department, (UPM).

All in all, my two years stay in UPM has contributed a lot to my growth as a scholar. I have been enriched with knowledge by attending lectures of outstanding professors in the university, and reading the up-to-date literatures available in the library. Meeting people from almost allover the world and sharing each other's cultures and norms has also been a great experience that I cherish forever. Last but not least, financial supports from the Agricultural Research Training Project of the Ethiopian Agricultural Research Organization (ARTP-EARO) are gratefully acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND NOTATIONS

2,4-D	2,4-Di-chlorophenoxy-acetic acid
ACN	Acetonitrile
ANOVA	Analysis of Variance
BAP	Benzyl Amino Purine
CB	Canada balsam
CE	Callogenesis Efficiency
CRD	Completely Random Design of experiments
CV	Coefficient of Variation
df	degree of freedom
DMRT	Duncan's Multiple Range Test
EAB	Ethanol (Absolute)
EAN	Ethanol (95%)
EC	Embryogenic Callus (calli)
ECE	Embryogenic Callus formation Efficiency
FAA	Formalin Acetic Acid
FRIM	Forestry Research Institute of Malaysia
g	gram
H&E	Hematoxylen and Eosin
HPLC	High Performance Liquid Chromatography
hrs	hours
IBA	Indole Butyric Acid
IEDC	Induced Embryogenic Determined Cells
IM	Induction Medium
IR	Infra Red Rays
Kn	Kinetin
LAF	Laminar Airflow Hood
µg	microgram
µL	Micro-liter
µm	micro meter
µM	micro molar (10^{-6}M)
masl	meters above sea level.
MB	Methylene blue
mg	milligram
mL	milliliter
MS	Murashige and Skoog's medium (1962). (salts, minerals and vitamins)
MSO	Murashige and Skoog's medium without any growth regulator
NAA	Naphthalene Acetic acid
NEC	Non Embryogenic Callus (calli)
OST	Optimum Subculturing Time
PEDC	Pre Embryonic Determined Cells
PGR	Plant Growth Regulators
PM	Proliferation Medium
rpm	rotation per minute
RT	Retention time

SAS	Statistical Analysis System
SCV	Settled Cell Volume
SE	Standard Error
SEM	Scanning Electron Microscopy
SV	Somaclonal Variation
TB	Toludine Blue
TBA	Tertiary Butyl Alcohol
TDZ	thidiazuron
UPM	University Putra Malaysia
UPW	Ultra Pure Water
UV	Ultra Violet rays
WPM	Woody Plant Medium

DEFINITIONS OF TERMS

Aseptic	Free from any microbial contamination
Auxin	A class of Plant Growth Hormones that control adventitious root initiation and micropropagation stages including callus formation
Azadirachtin	A natural insecticide that is abundantly found in the seeds of almost all species of the genus *Azadirachta* in the *Meliaceae* plant family.
Basal Medium	Aseptically prepared media containing the macro, micro and vitamins elements necessary for plant growth and development
Callogenesis	The process of inducing, proliferating, and maintenance of calli
Callus (Calli)	Tissue that develops as a response to injury caused by physical or chemical means; cells are differentiated but unorganized.
Clonal fidelity	The sameness of *in vitro* propagated materials to their mother tissue in all aspects of biology.
Column	A device used in HPLC as the bearer of the stationery phase
Conversion	Germination and hence regeneration of somatic embryos to plantlets (usually described in percentage)
Competence	A term that describes the potential of a cell to develop in a particular Direction (pathway) *in vitro*, such as an embryo or an organ
Cytokinin	A class of Plant growth hormones that stimulate cell division and initiation of shoot sequences in tissue culture
Dedifferentiation	A process in which specialized cells in a tissue become functionally undifferentiated. It is the temporal change in cells induced by a strong growth regulator action (usually auxin) or as a result of wounding or both.
Detachment	Removal of primary calli from its mother explant tissue and Subculturing it on fresh media.
Differentiation	Specialization of callus cells to become a known primordial pole (either shoot or root).

Explant	The plant part or propagules used to initiate the tissue culture process.
Exudation	The release of various phenolic compounds, from the explants, that oxidize to form browning of cultures (common feature in woody plant cultures)
Induction	Stimulation or orientation of plant cells or tissue to a particular development pathway, e.g. somatic embryogenesis
In vitro	Isolated from the living organism and artificially maintained in a controlled environment and nutritive media
In vivo	In the natural environment of the plant or in the plant itself
Injection	Loading of sample onto the HPLC bed to pump it to the column
Micropropagation	The production of plants from plant parts used *in vitro*.
Morphogenesis	The Origin and changes in the specific form (shape, structure, organization) during the development of explant cultures
Necrosis	Death of cultures explants or calli because of excessive oxidation or stress
Ontogeny	Stages of growth and development at different ages
Organogenesis	The *de novo* formation of organs, in particular roots and shoots either directly on the explant or indirectly from calli.
Plant Growth-Regulators	synthetic phytohormones of relatively low molecular weight that are active in small concentrations
Plantlets	Small complete plants that were produced via tissue culture
Polarity	Orientation in gravitational field (of shoots and roots).
Primordia	The earliest detectable stage of an organ, (root or shoot)
Regeneration	Development of callus cultures into whole plants or part
Recalcitrant Seed	Seeds that loose viability within a short period of time after dehiscence.(antonym= Orthodox seed)

Secondary-Metabolite	Bioactive natural compounds that have no known function in the basic physiological aspects of their source plant
Sentang	A vernacular name of *A. excelsa* in Malay language
Somatic cell	Vegetative cells having the complete genetic material (information) of the whole plant.
Somatic embryo	A rudimentary plant originating from somatic cell/tissue
Somatic-Embryogenesis	asexual developmental process that produces bipolar embryos (somatic) from previously differentiated or induced somatic cells.
Somatic Tissues	Tissues of asexual origin
Subculture	Transfer of parts or all of the culture into / onto a fresh medium to ensure sustainability of nutrient supply and perpetuation of the culture for its intended goals
Suspension	Plant cells grown in liquid cultures that is constantly agitated
Vitrification	Is a hyperhydricity characterized by a translucent, water-soaked or succulent appearance that can result in culture deterioration and failure to proliferate.
Tissue culture	The science of growing plant cells, tissues, or organs under aseptic conditions *in vitro*.
Totipotency	The concept that a single cell has the necessary genetic factors to reproduce all the characteristics of its mother plant.

CHAPTER 1

GENERAL INTRODUCTION

Sentang (*Azadirachta excelsa* [Jack] Jacobs) is a giant multipurpose tree species belonging to the genus *Azadirachta* in the *Meliaceae* family. The species is native to Southeast Asia, found in the lowland monsoon forests and dipterocarp rainforests. It is economically valued as source of food, fodder medicinal and pesticidal applications. Although it is much less investigated than neem tree (*Azadirachta indica* A. Juss), Sentang possesses insecticidal properties mainly because of the presence of natural insecticides in its seed kernels (Cui *et al.*, 1998). Hence, it is a valuable potential source of plant-based insecticides that need detailed investigations with regards to product maximization and large-scale utilization from its tissue culture.

Sentang is one of the priority tree species preferred for large-scale plantation (Anonymous, 2001). In recent years, the plant has been singled-out as a potential tree species to overcome timber deficits in its native areas. The tree has a straight trunk and high quality hard wood timber (Kijkar and Boontawe, 1995), which is not subject to damage by borers. Joineries and small-scale wood industries prefer Sentang wood to any light to medium weight hardwood. Due to this, it is intensively used for general construction, ceiling, window, door, furniture and carving purposes. These have limited its territory to most inaccessible and remote dipterocarp rainforest areas. Hence, there is a need to have large-scale plantation development of the species to meet the demands. In Thailand and Malaysia, there have been several efforts geared towards plantation development of Sentang (Anonymous, 1991; Ahmad and Mohd, 1997).

However, there are limitations that impede growers as well as investors willing to take part in plantation development of Sentang. The supply of quality planting materials is the main prerequisite in large-scale clonal forestry, and this is lacking in the case of Sentang. As a solution to this, clonal propagation of the species has been carried out via nodal cutting technique, although it has limitations. It is a multistep and labor-intensive process, which makes it commercially not competent as compared to other methods. Yet, the most critical snag against plantation development of the species is the loss of seed viability within short period of time after dehiscence (Norani, 1997). Although seeds can readily propagate Sentang, they are recalcitrant and hence cannot be stored for a long time. Propagation by seeds is also not preferred as it may cause unwanted genetic variability. Hence, techniques for mass propagation of the tree should be found.

Unlike the neem tree (*A. indica*), tissue culture has not been demonstrated as a viable strategy for propagation of Sentang, albeit the need for biotechnological approaches has been accentuated. However, the application of *in vitro* techniques might provide an avenue for a better use of the products from the tree species (timber, food, feed, and important secondary metabolites). For instance, tissue culture makes it possible for genetic transformation and *in vitro* preservation of Sentang germplasms, which in turn maximizes production of products including, *in vitro* production of important secondary metabolites (azadirachtin or other terpenoids and limonoids) and timber of high commercial values.