UNIVERSITI PUTRA MALAYSIA

PHYSALINS (13,l4-SECO-16,24-CYCLOSTEROIDS) PRODUCITON IN *PHYSALIS MINIMA* (LINN.)

JUALANG GANSAU

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PHYSALINS (13,14-SECO-16,24-CYCLOSTEROIDS) PRODUCTION IN *PHYSALIS MINIMA* (LINN.)

By

JUALANG GANSAU

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Science and Environmental Studies
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`IN THE NAME OF ALLAH, MOST GRACIOUS, MOST MERCIFUL'

Dedicated To:

My Parents
Father and mother: Mr. Gansau Dabiqsa and Ms. Rubiah Lumsten
Brothers and sisters: Ms. Nalaya, Ms. Nora, Mr. Dlasma@Maqlaa,
Ms. Jubaya, Ms. Ejinaya, Ms. Norpiah,
Mr. Gesorol and Mr. Mazli

My Wife: Loria @ Marlah Amirah Amani Abdullah
My Son: A'fif Iqkharuddin

To all my Teachers and Lecturers

Abstract of thesis presented to Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Doctor of Philosophy.

PHYSALINS (13,14-SECO-16,24-CYCLOSTEROIDS) PRODUCTION IN *PHYSALIS MINIMA* (LINN.)

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Chairman : Professor Hjh. Marziah Mahmood, Ph.D.

Faculty : Science and Environmental Studies

Physalis minima produces physalins, and these C_{28}-steroidal lactone compounds have great potentials in pharmaceutical industry. However, no detail information on the biosynthetic background of physalins in either intact plants or in cultured plant tissues. Therefore, this study was carried out to determine the physalins distribution in intact plants and in cultured plant tissues: callus, cell suspensions and hairy roots. Factors that control the growth and physalins production in plant tissue culture levels such as medium compositions, physical factors and precursors were also elucidated to improve the physalins productivity. The results showed that physalins accumulation in specific plant tissues of intact plants varied between 0.07 to 5.76 mg g^{-1} DW. Physalin contents increased two folds as the plant matured. Physalin A accumulated mostly in young fruits (3.82 mg g^{-1} DW), physalin B in young leaves (1.56-3.20 mg g^{-1} DW) and flower buds (2.88-3.60 mg g^{-1} DW), physalin D in flower buds (4.65-5.83 mg g^{-1} DW), physalin F in older leaves (4.51-9.89 mg g^{-1} DW), physalin J in immature and ripe fruit calyx (2.14-3.96 mg g^{-1} DW), and physalin N in young and old leaves (2.68-4.48 mg g^{-1} DW). In addition, the accumulation level of physalins in specific tissues was different among plants collected from different locations. In cultured plant tissues, the content of physalin B and F in hairy roots were

found to be higher (1.95-17.01 mg g^{-1} DW) than that in intact plants, but lower in callus (1.51-1.91 mg g^{-1} DW) and cell suspension (0.67-1.95 mg g^{-1} DW) cultures. Higher physalins production in callus and suspension cultures were obtained in cells derived from leaves followed by root and stem explants. Cell suspension and hairy root cultures were also capable of excreting physalins at lower concentration into culture medium. The study on the effect of medium compositions has shown that higher physalins production in callus, cell suspension and hairy root cultures were obtained in 1/2MS (half strength), MS (full strength) and B5 (full strength) basal media, each supplemented with 2.5, 3.5 and 3.5% (w/v) sucrose, respectively. An auxin-cytokinin interaction was observed to be important for callus cultures, as these two classes of phytohormones are required for higher growth and physalins production. Higher physalins production in callus culture was obtained in medium supplemented with a combination of 2,4-D and kinetin (9.0:4.5 μM). However, the addition of cytokinin in cell suspension culture appeared to stimulate irregular compact globular cells and growth of many root-like structures in the cell clumps. Higher physalins production in cell suspension was obtained in cultures supplemented with 9.0-18.0 μM NAA or 18.0 μM IAA. Meanwhile, in hairy root cultures, phytohormones often caused a growth disorganisation. The addition of 3-4 μM NAA increased the physalins production. Further investigations on hairy root cultures have shown that physalins accumulated mainly in mature part of root tissues. Inoculum of different root morphology did not significantly influence growth and physalins production. Meanwhile, the increase in number of inoculum root tips and medium volume resulted in changes of certain growth parameters. Hairy root cultures were capable to grow in pH values between 4.0-9.0, and higher physalins production was obtained at pH 5.0-7.0. Physalin productions in hairy roots also increased up to 1.2.1 folds when cultured under dark conditions supplemented with alanine, leucine and valine.

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PENGHASILAN FISALIN (13,14-SEKO-16,24-SIKLOSTEROID) DALAM *PHYSALIS MINIMA* (LINN.)

Oleh

JUALANG GANSAU

January 2001

Pengerusi : Profesor Hjh. Marziah Mahmood, Ph.D.

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Pokok *P. minima* telah menghasilkan kompaun steroid lakton C_{28} yang dinamakan fisalin dan mempunyai potensi dalam industri farmaseutikal. Walau bagaimanapun, tiada kajian secara mendalam mengenai biosintesis fisalin dalam pokok induk atau dalam kultur tisu. Oleh yang demikian, kajian telah dijalankan ke atas pokok induk dan kultur tisu bagi kalus, sel ampaian, dan akar transgenik untuk menentukan taburan penghasilan fisalin. Faktor yang mempengaruhi tumbesaran dan sintesis fisalin dalam kultur tisu seperti komposisi medium, faktor fizikal dan bahan pemula juga dibincangkan untuk meningkatkan hasil fisalin. Keputusan kajian menunjukkan fisalin dikumpulkan dalam tisu pokok induk dalam nisbah kepekatan tertentu (0.07-5.76 mg g^{-1} DW). Kandungan fisalin juga didapati meningkat dua kali ganda dengan perubahan pematangan tumbesaran pokok. Fisalin A kebanyakannya dikumpul dalam buah muda (3.82 mg g^{-1} DW), fisalin B dalam daun muda (1.56-3.20 mg g^{-1} DW) dan kudup bunga (2.88-3.60 mg g^{-1} DW), fisalin D dalam kudup bunga (4.65-5.83 mg g^{-1} DW), fisalin F dalam daun tua (4.51-9.89 mg g^{-1} DW), fisalin J dalam kalik buah masak dan matang (2.14-3.96 mg g^{-1} DW), dan fisalin N dalm daun muda dan tua (2.68-4.48 mg g^{-1} DW). Namun begitu, kandungan fisalin dalam tisu tertentu didapati berbeza pada pokok yang diambil dari lokasi yang berlainan. Dalam

kultur tisu, penghasilan fisalin B dan F dalam akar transgenik didapati lebih tinggi (1.95-17.01 mg g^{-1} DW) dari pokok induk, dan rendah dalam kalus (1.51-1.91 mg g^{-1} DW) dan sel ampaian (0.67-1.95 mg g^{-1} DW). Penghasilan tertinggi fisalin dalam kultur kalus dan sel ampaian telah diberikan oleh sel dari daun berbanding dengan sel dari batang atau akar. Kultur sel ampaian dan akar transgenik juga berupaya pada kepekatan rendah untuk membebaskan fisalin ke dalam media kultur. Kajian pada kesan komposisi medium menunjukkan penghasilan tertinggi fisalin pada kultur kalus, sel ampaian dan akar transgenik masing-masing diperolehi dalam media asas 1/2MS, MS dan B5 yang ditambah dengan 2.5-3.5% (w/v) sukrosa. Interaksi auksin-sitokinin didapati penting bagi pertumbuhan kalus dan memberikan hasil fisalin yang tinggi. Kandungan fisalin dalam kalus didapati tinggi dalam medium yang ditambah kombinasi 2,4-D-kinetin. Penambahan sitokinin dalam kultur sel ampaian didapati mengaruh pembentukan sel bulat yang keras dan pertumbuhan struktur seperti akar pada sekeliling sel. Penghasilan tertinggi fisalin dalam kultur sel ampaian diperolehi dalam kultur yang ditambah dengan NAA atau IAA. Penambahan pengawal atur pertumbuhan pada kultur akar transgenik didapati merangsang ketidaktentuan pertumbuhan akar dan akhirnya menurunkan berat tumbesaran. Namun begitu, penambahan NAA berupaya meningkatkan penghasilan fisalin. Kajian lanjut pada kultur akar transgenik menujukkan fisalin lebih banyak dikumpulkan pada bahagian akar yang telah matang. Morfologi akar yang berbeza yang digunakan sebagai pemula kultur didapati tidak berbeza dari segi keupayaan tumbesaran dan penghasilan fisalin. Perubahan bilangan akar dalam kultur pemula dan isipadu media yang digunakan juga tidak secara berkesan mempengaruhi sintesis fisalin, tetapi didapati mengubah beberapa parameter pertumbuhan. Akar transgenik juga berupaya untuk tumbuh pada julat pH media antara pH 4.0-9.0, dan hasil fisalin tertinggi didapati pada julat pH 5.0-7.0. Fisalin dalam akar transgenik juga boleh ditingkatkan sehinga 1.2-2.1 kaliganda bila ditumbuhkan dalam keadaan gelap dan dengan penambahan asid amino (alanine, leucine dan valine).

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LIST OF ABBREVIATIONS

%	Percentage
^0C	Degree Celsius
1IAA	1 mg L^{-1} indole-3-acetic acid
2,4-D	2.4-Dichlorophenoxyacetic acid
BAP	6-benzylaminopurine
$CHCl_3$	Chloroform
cm	Centimetre
CoA	Co-enzyme A
conc.	Concentration
ctrl	Control
cul.	Culture
d	Day
dH_2O	Distilled water
Dicamba or Dic	3,6-Dichloro-o-aniscic acid
DMAPP	3,3-Dimethylallyl pyrophosphate
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid (ferric sodium salt)
e.g.	Example
EtOH	Ethanol
fruc	Fructose
FW	Fresh weight
FPP	Farnesyl phyrophosphate
g	Gram
gluc	Glucose
GPP	Geranyl phyrophosphate
h	Hours
H_2SO_4	Sulphuric acid
HMG-CoA	3-Hydroxy-3-methylglutyrl-Coenzyme A
HR	Hairy root
i.e.	That is
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
IPP	Isopentenyl pyrophosphate
αKIA	α-Kitoisocaproate
Kinetin or Kin	6-furfurylaminopurine
L	Litre
LR	Lateral branches
MC-CoA	3-methylcrotonyl-coenzyme A
MCCase	3-methylcrotonyl-coenzyme A carboxylase
MeOH	Methanol
mg	Milligram

$MgCl_2$	Magnesium chloride
MG-CoA	3-Methylglutyrl-CoA
min	Minute
mL	Millilitre
MVA	Mevalonic acid
Na_2EDTA	EDTA disodium salt
$Na_2EDTA\text{-}2H_2O$	Na_2EDTA dihydrate
NAA	α-Naphthaleneacetic acid
NaOH	Sodium hydroxide
NBT	Nitro tetrazolium blue
nd	Not determine
NT	Normal root or non-transformed root
O_2	Oxygen
ORF(s)	Open reading frame(s)
PGR(s)	Plant growth regulator(s)
Picloram or Pic	4-Amino-3,5,6-trichloropicolinic acid
R_f	Distance of the substance over distance of the solvent movement
rol	Rooting locus
rpm	Revolution per minute
R_T	Retention time (min)
SDS	Sodium dodecyl sulphate
suc	Sucrose
t_d	Doubling time (d)
v/v	Volume for volume
w/v	Weight for volume
N	Normality
μ	Specific growth rate (d)
µg	Microgram
µM	Micromolar

CHAPTER 1

INTRODUCTION

Plants are widely known as superb synthesisers of 'natural products'. These compounds, also called as 'secondary plant products', which are low molecular weight and often restricted to special plant families or even genera. They are not important for the primary metabolism of the plant, but in many cases of great importance for the plants to survive in its environment (Farnsworth, 1985; Alfermann and Petersen, 1995). Plant secondary products are used extensively in commerce and trade especially between countries, particularly in the food additives, nutraceutical and pharmaceutical industries. The use of plant-based medicines either as natural drugs or herbal remedies varies greatly among countries. Recent surveys estimate that over 80% of the population in parts of the developing world still rely on plant-derived medicines for their primary health cares and food supplements (Simmonds and Grayer, 1999). Meanwhile, total world trade of medicinal plants in 1980 only, was in excess of US$ 551 million (Pillipson, 1990). The trade in plants used within Europe for non-conventional medicines is increasing by 15-20% a year, with an import value of US$3.6 billion in 1995 (Simmonds and Grayer, 1999). Thus, plants are still as the main immediate source of medicine available to the majority of people in the world. It has been estimated that 20-30% of the world's flora of 250,000-500,000 species have been subjected to phytochemical and pharmacological investigations (Simmonds and Grayer, 1999). Herbal and medicinal plants have still to be collected from the wild and

some sources are locally planted or cultivated (e.g. garlic, ginger, and ginseng) (Phillipson, 1990).

The majority of plant natural products used medicinally are terpenoids (mono-, sesqui-, di-, tri-, steroids, cardenolides), quinones, ligans, flavonoids, alkaloids and saponins. Some of these compounds cannot be synthesised in laboratories. Compounds that possess interesting bio-pharmacological or other biological properties and consumed in large quantities required more detailed investigations. Parent plant materials are not always available and some are endangered due to severe over collection. Therefore, plant tissue culture techniques offer an alternative source for the production of such compounds. *In vitro* cultures lead to the possibility of harvesting the desired natural products everywhere in the world without contamination of pesticides, herbicides or insecticides, and also to overcome the natural heterogeneity in plant material and variations in product content (Taticek *et al.*, 1991). There have been a number of reports on using plant tissue and organ cultures to produce a wide range of secondary compounds (Zenk, 1977; Staba, 1980; Rhodes *et al.*, 1990,1997). However, plant callus, cell suspension and hairy root cultures are the common plant tissue culture systems that have been adopted by many researchers as compared to other cell or organ cultures (Su, 1995; Hamill and Lidgett, 1997). Additionally, those plant culture systems (cell suspension and hairy root cultures) can potentially grow in the bioreactor, which is much quicker than from plants grown in the field (Alfermann and Petersen, 1995).