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Bioactivity and Biochemical Evaluation of Genetically Modified *Mitracarpus hirtus* L.

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CONTENTS

LIST OF ABBREVIATIONS

bp	base pair
°C	degree Celsius
CPPU	2-chloro-4-pyridyl-N-phenylurea
cm	centimeter
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DW	dry weight
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
e.g.	Exempli gratia (Latin), for example
et al.	et. alli (Latin), and other
FCM	flow cytometric analysis
FW	fresh weight
g	gram
$g.L^{-1}$	gram per liter
h	hour
HPLC	high performance liquid chromatography
i.e.	id est, that is
kbp	kilobase pair
L	liter
LA	Luria-Bertani Agar medium
LB	Luria-Bertani medium
M	Molar
MeJ	Methyl Jasmonate
mg	milligram
$mg.L^{-1}$	milligram per liter
µg	microgram

MH	Mueller Hinton
MIC	minimal inhibitory concentration
min	minute
ml	milliliter
µl	microliter
mm	millimeter
mM	millimolar
µm	micrometer
µM	micromolar
µmol m^{-2} s^{-1}	micromole per square millimeter second
MS	Murashige and Skoog
NaCl	sodium chloride
nm	nanometer
OD	optical density
PCR	polymerase chain reaction
PGR	plant growth regulator
pH	power of hydrogen ion
ppm	part per million
PVP	Polyvinylpyrrolidone
DAPI	4′, 6′-diamidino-2-phenylindole
Ri T-DNA	Root inducer transfer deoxyribonucleic acid
rpm	revolutions per minute
sec	second
T-DNA	transfer deoxyribonucleic acid
TE	Tris-EDTA buffer
Tris	Tris (hydroxymethyl) aminomethane
Tris–HCl	hydroxymethyl aminomethane hydrochloride
UV	ultraviolet
V	volt

v	volume
w/v	weight by volume

CHAPTER 1

General introduction

1.1 General botanical information of *Mitracarpus hirtus* and related species

Mitracarpus hirtus belonging to family Rubiaceae is herbaceous annual weed and easily spread in garden and farm. It is distributed throughout neotropical and tropical regions such as United States of America, India, Malaysia, Myanmar and Thailand. *M. hirtus* has been described as approximately 40 cm tall, opposite leaves with 2-6 cm length, 0.5-2 cm width, white flower in dense axillary clusters, and pale yellowish brown seeds with ellipsoid-rectangular (Fosberg et al. 1993). In medicinal approach, it was mentioned to use for treating antidotes (e.g. venomous stings, and bites), oral or pulmonary troubles. In phytochemistry, it was reported as antibiotic, bacteriostatic fungistatic, and insecticides. However, the application of *M. hirtus* was not extensively investigated.

The other interesting related species, *Mitracarpus scaber* Zucc, has been studied focusing on its phytochemical constituent. The investigation revealed the presence of alkaloids, tannins, cardiac glycosides and saponins in the leaves of this plant (Abere et al. 2007). The essential oil of this plant provided the antimicrobial and cytotoxic activity against *Bacillus cereus* (MIC = 625 μg ml^{-1}), *Aspergillus niger* (MIC = 313 μg ml^{-1}) and marginal cytotoxic activity on MCF-7 cells (58.7±7.6% kill at 100 μg ml^{-1}) (Owolabi et al. 2013). Antibacterial and antimycotic activities of *M. scaber* were investigated and consequently the extract was fractionated leading to isolation of interesting compounds. Among these compounds, gallic acid and 3,4,5-trimethoxybenzoic acid were shown to inhibit the growth of *Staphylococcus aureus*. The inhibition of *Candida albicans* was observed when tested with isolated 4-methoxyacetophenone and 3,4,5-trimethoxyacetophenone (Bisignano et al. 2000). *M. scaber* had antihepatotoxic potential as it affected carbon tetrachloride-induced acute liver damage in the rat. Treatment with

M. scaber decoction resulted in hepatoprotection against CCl4-induced liver injury (Germano et al. 1999). Methanolic extract derived from *M. scaber* leaves, inflorescences and roots showed inhibition against *Dermatophilus congolensis* at concentration of 3 µg ml^{-1} while stem extract was effective at 5 µg ml^{-1} (Imam et al. 2008). Methanolic (80%) and n-hexane extract gave anti-bacterial, yeast, and fugal activity (Cimanga et al. 2004). Essential oil of the other *Mitracarpus*, *M. frigidus*, containing linalool and eugenol acetate as major component exhibited an anti-bacterial and antifungal activities against *Staphylococcus aureus*, *Bacillus cereus, Pseudomonas aeruginosa,* and *Enterobacter cloacae* (Fabri et al. 2012).

To date, plants are important sources of useful compounds (Berdy 2005). Only 15% of 300,000 plant species have been phytochemically investigated, and some of them were screened for bioactivity (Verpoorte 2000). Therefore, it is important to evaluate the new screening plants to use as the novel sources of useful metabolites. *M. hirtus* is an alternative potential one, but it should be characterized for its phytochemical compounds and bioactivity.

1.2 Production of polyploid plant

Polyploid induction in plants was established in an attempt to create the new characteristics especially for medicinal purpose and commercial competition. For example, larger leaves and flowers are required by orchid and plant market. Polyploid induction plays important role in the hybridization of orchid because it can help to restore the fertility by doubling the chromosome number and allo-tetraploids production. In pharmaceutical approach, polyploid plants were produced to increase the production of important secondary metabolites for use as a medicine. Polyploidy in plant was induced by natural or synthetic compounds which temporarily interrupt the cellular cytoskeleton during mitosis. The well-known and widely used chemical compound is colchicine (Fig. 1 appendix) because of its effectiveness in plant genetic engineering as shown in previous studies (Banyai et al. 2010; Jesus-Gonzalez and Weathers 2003; Kim et al. 2004; Lin et

al. 2011). Colchicine, a toxic chemical, was classified as a teratogen (a substance causing birth defects) and has a potential to be carcinogen. It is capable to alter genetic material by preventing the microtubule formation or disturbing the mitotic spindle formation, which arranges and tracks the chromosome during cell division. The chromosomes are arrested and results in multiple set of chromosomes in one cell. In addition, the chromosomal defect could occur because of its acute toxicity.

Enhancements of secondary metabolite production in plant were observed in several polyploid plants. In the roots of *Panax ginseng*, octoploid showed higher content in Rg-group of ginsenosides than the original natural tetraploid (Kim et al. 2004). Artemisinin content in *Artemisia annua* tetraploids showed higher level than the diploid clone as observed in many reports (Banyai et al. 2010b; Jesus-Gonzalez and Weathers 2003; Lin et al. 2011).

1.3 *Agrobacterium rhizogenes* as a useful tool for plant genetic engineering and hairy root production

Transformation using *Agrobacterium*-mediated method is an alternative tool for genetic engineering in plant. It provides the advantages as low copy number, large size of foreign DNA transfer, efficient method, adaptation to different cell types and the transformants are mitotically and meiotically stable (Gelvin 2003; Riva 1998). *A. rhizogenes*, gram negative soil born bacterium, induces hairy root formation by following 4 steps: 1) chemotactism which induces bacterial movement to the plant cells, 2) binding of bacteria with surface component of plant cell wall, 3) activation of virulence (*vir)* genes in bacteria, and 4) transfer, integration, and expression of transfer-DNA (T-DNA) from the root-inducing (Ri) plasmid into a plant genome (Zupan and Zambryski 1997). Genetic information of *Agrobacterium* infection is contained in Ri plasmid and only the right and left T-DNA (T_R-DNA and T_L-DNA) regions are transferred and integrated into the plant genome. Gene of T_L-DNA synthesizes compounds which recruit cell differentiation into roots by the influence of endogenous auxin synthesis (Giri and Narasu

2000; Ooms et al. 1986; Shen et al. 1988). The Ri-plasmid contains *rolA, B, C*, and *D* genes at T_L-DNA (White et al. 1985) which are responsible for root formation in transformed cell (Bensaddek et al. 2008, Christey and Braun 2005).

Hairy root, the differentiated culture, is suitable for secondary metabolite production in bioreactor system. It has various characteristics as follows: ability to grow in plant growth regulator free-medium, high mass production, high potential for elicitor application, phytoremediation, biochemical and genetic stability, being available for studying the biosynthetic pathways of interesting compounds and appropriate for genetical modification and manipulation when compared with other cell culture systems. Hairy roots were induced in many plants such as *Artemisia annua* (Xie et al. 2000), *Panax ginseng* (Yu et al. 2005), *Ammi majus* (Krolicka 2001), and *Atopa belladonna* (Bonhomme et at. 2000; Srivastava and Srivastava 2007). Plant based-vaccine and other medicinal compounds were produced by hairy root culture system. Hepatitis B surface antigen was expressed in potato hairy roots (Kumar et al. 2006). Hairy roots of *Gmelina arborea* Roxb were induced for production of verbascoside, a phenylpropanoid glycoside of medicinal value (Dhakulkar et al. 2005). Moreover, this system is effective for transgenic plants production as observed in *Vitis vinifera* L. (Nakano et al. 1994). Hairy root system is one important source of new secondary metabolite production discovery. A new sesquiterpene; (Z)-7-acetoxy-methyl-11-methyl-3-methylenedodeca-1,6,10-triene (AMDT); was isolated and identified from the methanol extract of the hairy root culture of *Artemisia annua* (Zhai et al. 2010a). Therefore, the hairy root induction in *M. hirtus* is an interesting system for further study in biosynthesis of secondary metabolites and industrial application.

1.4 Enhancement of secondary metabolite by chemical and stress treatments

Elicitation is a potential strategy to enhance plant metabolite production using physical, biological or chemical elicitors. Various studies reported the application of elicitors on hairy roots leading to increasing of metabolite production. Mycelial extracts

from endophytic fungus *Colletotrichum sp.* stimulated the artemisinin content in the hairy roots of *Artemisia annua* from 0.8 mg g^{-1} DW to 1 mg g^{-1} DW after treatment (Wang et al. 2001). Among various types of elicitor, chemical elicitors have been successfully applied to many plants according to their practicality and effectiveness. Methyl jasmonate (MeJ) stimulated the metabolite production in many plants such as alkaloids, phenolics and diterpene glycosides in *Nicotiana attenuata* (Keinanen et al. 2001), antioxidant activity and flavonoid content in blackberries *Rubus sp.* (Wang et al. 2008), gums and anthocyanin in peach (Saniewski et al. 1998), and tepenoid in *Picea abies* L. (Martin et al. 2002). MeJ and salicylic acid (SA) increased isoflavonoid production in *Pueraria candollei* hairy root cultures (Udomsuk et al. 2011). Total production of isoflavones in 30 day-old hairy root culture of soybean was enhanced up to 10.7 and 5.8 folds after treatments of 100 μM MeJ for 72 h or 200 μM SA for 96 h, respectively (Theboral et al. 2014). Although MeJ and SA are popular to use as elicitor in many reports, they also affected to reduce plant cell mass too (Theboral et al. 2014). Therefore, the effect of other alternative chemical elicitors should be investigated. Among these, synthetic bioregulator that has its action like phytohormone such as a group of cytokinin is interesting. It plays important roles not only on plant growth and development but also on metabolite productions, as one of key hormones sharing its related biosynthetic pathway with terpenoid compounds. Thereby, alteration of cytokinin production or accumulation in plant either by internal interference in metabolic biosynthesis or by external application of abundant synthetic cytokinin to plant cultures may regulate the change in growth characters as well as secondary metabolites production. The previous reports demonstrated that overexpression of isopentenyl transferase (*IPT*), a cytokinin biosynthetic gene resulted in enhancement of both endogenous cytokinin (2-3 folds) and the sesquiterpene compound (artemisinin) up to 30-70% compared with the control plants (Sa et al. 2001). On the other hand, terpenoid indole alkaloids content in *Catharanthus roseus* was increased after exogenous treatment of tran-zeatin (Papon et al. 2005). Cytokinin was then used as elicitor to increase secondary metabolite production in several plants. The total yield of essential oil produced in peppermint (*Mentha piperita*) was 40%

increased by addition of cytokinin (N6-benzylaminopurine, BAP) solely. Cytokinin types and concentrations influenced not only on shoot proliferation but also on the *in vitro* production of bioactive secondary metabolites of *Aloe arborescens* Mill (Amoo et al. 2012). For exogenous treatment, a form of non-purine cytokinin is more effective and practically used. The CPPU (2-chloro-4-pyridyl-N-phenylurea) (Fig 2, appendix) is a type of non-purine synthetic cytokinin which affects cell division, differentiation and plant development. Therefore, in the present study, effect of CPPU on variation of plant secondary metabolites in *M. hirtus* was demonstrated using hairy root culture system.

1.5 Objectives of the study

Mitracarpus hirtus L., common herbaceous weed, was mentioned to have wide usages such as antidotes insecticide, and treatment from parasitic infection. According to these characteristics, it was interesting to investigate this plant to use as an alternative pharmaceutical source. As mentioned, the study of this plant was conducted according to the following purposes;

1. To establish the tetraploid plants of *M. hirtus* and compare the anatomy, morphology, phytochemical constituent and bioactivity of the tetraploid plants with original diploid plants

2. To establish the *M. hirtus* hairy root culture via transformation using *Agrobacterium rhizogenes* A13

3. To investigate the effect of CPPU elicitation on phytochemical contents in hairy root culture of *M. hirtus.*

1.6 Outline of dissertation

This dissertation comprises of four chapters. Chapter 1 described a general introduction about *Mitracarpus hirtus* and other related species, production of polyploid plant, *Agrobacterium rhizogenes* as a useful tool for plant genetic engineering and hairy root production, and enhancement of secondary metabolite by chemical treatments.

Tetraploid induction of *Mitracarpus hirtus* by colchicine treatment was presented in chapter 2 including, growth characterization of diploid and tetraploid plants. Antibacterial activity and analysis of biochemical compounds derived from methanolic extract of diploid and tetraploid plants were analyzed using gas chromatography mass spectrometry (GC–MS) and Hierarchical Clustering method. In chapter 3, hairy roots of *M. hirtus* were induced by *Agrobacterium rhizogenes* A13 and further confirmed for the *rol* gene expression. Growth and proliferation of hairy root cultures were studied and consequently the effect of CPPU on biochemical constituents of *M. hirtus* hairy root was investigated. The results were discussed and concluded in chapter 4 and all issues were summarized again in summary part.

CHAPTER 2

Tetraploid induction of *Mitracarpus hirtus* L. by colchicine and its characterization including antibacterial activity

2.1 Introduction

The presence of bioactive compounds in plants can vary due to a number of factors. These include genotype, climate, geography, harvest period, and interval of storage (Maison et al. 2005; Pholphana et al. 2004; Prathanturarug et al. 2007). Therefore, genetic improvement plays an important role to increase active content in plant species. Polyploid induction has been shown to be an effective tool to genetically enhance secondary metabolite production (Banyai et al. 2010; Dhawan and Lavania 1996). Polyploidy can be induced by chemical compounds such as colchicine, which inhibits chromosome segregation during cell division, leading to chromosome doubling. In *Panax ginseng*, colchicine induced octoploids showed higher concentrations of Rg-group ginsenosides than the roots of the original tetraploids (Kim et al. 2004). In *Artemisia annua*, content of artemisinin, an important anti-malarial agent, was compared between a diploid hairy root line and tetraploid lines induced from the diploid line after treatment with colchicine. The results showed that in the tetraploid clones artemisinin levels were up to six times higher than those found in the diploid clone (Jesus-Gonzalez and Weathers 2003). Higher artemisinin yields were also detected in tetraploid plants of *A. annua*, which showed concentrations 1.5 times higher than those for diploid plants at the full blooming stage (Banyai et al. 2010). Higher levels of artemisinin content in tetraploids over diploids was also demonstrated in *A. annua* by Lin et al. (2011). Plants are evaluated as the important sources of useful metabolites (Berdy 2005). While there are upwards of 300,000 plant species worldwide, it is estimated that only 15 % have been evaluated phytochemically, and 6 % screened for biological activity (Verpoorte 2000). With such a low percentage of species screened for bioactive compounds, there is a need and value in screening and

evaluating new germplasm. *Mitracarpus hirtus* L., is a common weed belonging to the Rubiaceae family. It is widely distributed as it easily spreads in gardens, farms and fields in neotropical and tropical regions. It has been reported that *M. hirtus* possesses medicinal value, commonly used as an antidote for stings and bites, an antibiotic, an insecticide, and for treatment of cutaneous parasitic infection (Burkill 1970). Studies looking into the pharmaceutical properties of this plant, however have been limited. To date, studies have focused on the phytochemical screening of the related species, *Mitracarpus scaber* Zucc., finding alkaloids, saponins, tannins, and cardiac glycosides present (Abere et al. 2007). Extracts from *M. scaber* have been shown to possess antibacterial and antifungal activity (Cimanga et al. 2004; Owolabi et al. 2013). Due to these finding in *M. scaber*, and the reported medicinal properties of *M. hirtus*, it is therefore considered likely that *M. hirtus* also possesses pharmaceutically active compounds of interest. With the studies on ploidy manipulation in *A. annua*, and the resulting changes in secondary metabolite production, *M. hirtus* makes for an interesting candidate to both evaluate plant compounds for bioactivity and assess the impact of ploidy on secondary metabolite production on compounds of interest. In the present study, colchicine induced tetraploid plants of *M. hirtus* exhibiting phytochemical activity against an infectious pathogen (*S. aureus*) has been successfully demonstrated. Moreover, characterization of plant anatomy, morphology, antibacterial activity, and biochemical constituents of crude extract were compared between tetraploid and diploid wild type.

2.2 Materials and methods

2.2.1 Plant materials

Plant material used in this study was *Mitracarpus hirtus* L. The excised 1 cm shoots from *ex vitro* plants (selected from Bangkok, Thailand) were surfaced–sterilized with 0.5 % sodium hypochlorite solution (Clorox®) and cultured on 0.7 % agar-solidified MS medium (Murashige and Skoog 1962) with 3 % sucrose and incubated under 25 ± 2 °C, 60 ± 5 % relative humidity and 16 h photoperiod with photosynthetic photon flux

density (PPFD) of 60 ± 5 µmol m^{-2} s^{-1}. Shoots with 2–3 nodes from sterilized cultures were monthly excised and micro-propagated on the same medium for proliferation.

2.2.2 *In vitro* colchicine treatment of *M. hirtus*

Leafless shoots sized 1 cm were immersed in filter-sterilized 0, 0.01, 0.1 and 1 % w/v colchicine solutions for 24 h at room temperature of 25 ± 2 °C. Soaked shoot-meristems were washed with MS liquid medium before blotting dry on sterilized tissue paper. Treated meristems were cultured and multiplied on 0.7 % agar-solidified MS medium before confirmation of ploidy level by cytological analysis.

2.2.3 Flow cytometric (FCM) analysis

Ploidy level of the *in vitro* plants obtained after colchicine treatments was analyzed by flow cytometry (Partec PA cytometer equipped with a mercury lamp, Partec, Germany) according to the method of Banyai et al. (2010). In order to extract plant nuclei, 0.1 g fresh weight of plant leaves were collected and chopped by blade in a small Petri dish with 0.25 ml of solution A of plant high resolution DNA kit type P (Partec, Germany). After incubation at room temperature for 5 min, 1.0 ml of staining solution (10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1 % (w/v) Polyvinylpyrrolidone (PVP), 0.1 % (v/v) Triton X-100 and 2 mg l^{-1} 4′,6′-diamidino-2-phenylindole (DAPI), pH 7.5) was added and filtrated through a nylon mesh with pore size of 30 µm. After staining, sample solutions were analyzed by flow cytometry for determination of relative nuclear DNA content.

2.2.4 Determination of chromosome number

Six months after excised shoots were treated in colchicine and routinely sub-cultured onto hormone-free MS medium every 30 days, the putative mutants were briefly screened by FCM as mentioned above and further analyzed for chromosome numbers using squash technique. Root tips (approximate 0.5–1.0 cm long) were cut from roots of plantlet showed FCM positive as diploid and tetraploids. Root tips segments were

incubated in 2 mM 8-hydroxyquinoline at 20 °C. After incubation for 6 h, root tips were washed with distilled water and fixed in a mixture of absolute ethanol and glacial acetic acid (3:1) for 30 min at 4 °C. Root tips were washed with distilled water and hydrolyzed with 1 N HCl for 3 min at 65 °C. After removing HCl solution, root tips were washed with distilled water and staining with 1–2 % acetocarmine at least for 30 min. Treated samples were excised 1–2 mm long and squashed on slide glass, dropped with 45 % acetic acid-glycerol (9:1), and covered with cover glass. At least ten cells with metaphase chromosomes were analyzed by observing under microscope (magnification as 400–1,000×).

2.2.5 Characterization of guard cells

In vitro leaves were collected from diploid (WT1), and tetraploid (CC102, CC110) plants. Leaves were sampling, each from individual plant of each line for total 8 replications (1 leaf per 1 replication). The lower epidermis was glazed with nail varnish and air dried for 15–30 min. Dried film of nail varnish was peeled with tape and stuck on slide before observation of the number of guard cell and characteristics under microscope.

2.2.6 Plant cultivation and transplantation

In vitro plants of diploid (WT1) and tetraploid (CC102 and CC110) *M. hirtus* were maintained on 0.7 % agar-solidified MS medium supplemented with 3 % w/v sucrose at 25 ± 2 °C, 60 ± 5 % relative humidity and 16 h photoperiod with PPFD of 60 ± 5 μmol m^{-2} s^{-1}. Newly emergent shoots with 2–3 nodes of each plantlet were excised and subsequently sub-cultured for multiplication using these culture conditions every 30 days for 8 months. Diploid and tetraploid plants were then acclimatized under photoautotrophic environments using vermiculite as supporting material supplemented with MS (sugar free) liquid medium. In each diploid (WT1) and tetraploids (CC102, CC110), ten plants were transplanted into the pot after acclimatization for 2 weeks. Plant height, number of shoots, number of leaves, and fresh and dry weights of leaves were measured after culture for 3 months.

2.2.7 Methanolic extraction and preparation for antimicrobial activity test

Leaves of both diploid and tetraploid *M. hirtus* were collected 3 months after transplantation and overnight dried at 50 °C. Dried leaves were ground with liquid nitrogen before extraction with methanol. Methanolic extracts were filtrated through Whatman® No. 1 filter paper before removing the solvent out by rotary evaporation at 40 °C (Buchi Rotary Evaporator®). Crude extracts were then redissolved with dimethyl sulfoxide (DMSO) to make 100 $\mu g.\mu l^{-1}$ stock solution and kept at -20 °C before antimicrobial analysis.

2.2.8 Antimicrobial activity test against bacteria

Screening of candidate microbes to be used with plant extracts was preliminarily performed using the representative microbes Gram negative (*Escherichia coli*), Gram positive (*Bacillus subtilis*) bacteria, and yeast (*Candida albican*). The methanolic extracts of *M. hirtus* WT1 showed no bioactivity against *C. albican* and *E. coli* but showed a mild effect on Gram positive *B. subtilis* (data not shown). As *Bacillus subtilis* is identified as a non-pathogenic bacterium, the bioactivity of methanolic extracts against human pathogenic bacteria was tested in order to identify the possible pharmaceutical properties of *M. hirtus*. This was done using the Gram positive bacteria *Staphylococcus aureus*. The bacterial solutions were refreshed from -80 °C stock cultures by streaked onto agar plate of Luria Agar (LA) medium. A single colony of each bacterial strain was picked up and inoculated into a 125 ml flask containing 30 ml liquid Luria Broth (LB) media. They were shaken (120 rpm) under 37 °C for 16–18 h prior to dilution to get a final concentration of 0.1 OD_{600}. Pour plate technique was applied by mixing 1 ml of bacterial solution with 9 ml of Mueller–Hinton (MH) medium in a plate of 90 mm diameter. For antibacterial activity test, disc diffusion method was applied by dropping 20 µl (volume) of plant crude samples (each at 100 $\mu g.\mu l^{-1}$) on sterilized disc papers (6 mm diameter) in order to get the final concentration of each tested crude at concentration 2 mg/disc. The paper disc soaked with DMSO without plant extract was used as negative control and the antimicrobial activity were compared between extracts derives from

diploid and tetraploid plants. Tested disc soaked with plant crude was allowed to dry at room temperature in laminar flow cabinet. Paper discs (total 7 replications per treatment, 1 paper disc for 1 replication) containing crude extracts were placed onto bacterial plates and incubated overnight at 37 °C before measuring the inhibition zone (mm). The antibacterial evaluation was examined according to the inhibition zone that observed at 8, 16 and 24 h.

2.2.9 Gas chromatography mass spectrometry (GC–MS) analysis

The biochemical constituents in the methanolic extracts of diploid and tetraploids *M. hirtus*, each at concentration 10 mg ml^{-1} were analyzed using gas chromatography mass spectrometry (GC–MS)-based metabolite profiling with HP5-MS capillary fused silica column (30 m length, 0.25 mm I.D., 0.25 lm film thickness), Agilent Technologies, USA. The oven temperature program was set as follows: initially at 50 °C, raise 10 °C/min to 280 °C. The GC–MS interface temperature was set at 280 °C. Extracts were injected with split ratio 50:1, flow rate at 1 ml min^{-1}, mass range from m/z 35–600 (modified from Kalaivani et al. 2012; Owolabi et al. 2013; Roy et al. 2010). Internal standard in this study was methyl heptadecanoate (C17). The detected compounds were searched base on the NIST-08 (National Institute of Standards and Technology) library.

2.2.10 Hierarchical clustering analysis (HCA) of phytochemical analysis with its antibacterial activity.

The detected compounds derived from GC–MS analysis were clustered with antibacterial activity by hierarchical clustering analysis (HCA) as previously describe in grouping technique (Sumner et al. 2003). This technique builds a hierarchy of cluster by grouping the data set using Pearson correlation (average linkage clustering) by multiexperiment viewer (MeV) software, version 4.9 (Saeed et al. 2003) to analyze, visualize and determine the data-mining of overall data set. The output showed the relation between detected compounds and antibacterial activity as heat map with the degree of difference and indicated the correlation by the branch length.

2.3 Results and discussion

2.3.1 Tetraploid induction by colchicine treatment

In the present study, the excised shoot apices of *M. hirtus* treated with colchicine at concentrations of 0, 0.01, 0.1 and 1 % (w/v) were cultured on agar-solidified MS medium and the survival percentage was observed after 1 month of treatment. The colchicine concentration at 1 % (w/v) caused 100 % death of shoot meristems while all of those treated with the other concentrations survived and grew into plants as shown in Table 1. Survival shoots were subsequently subcultured every 30 days in this media condition and preliminary screened for the putative tetraploid by flow cytometric (FCM) analysis after 3 months. The relative nuclear DNA content in leaves of each plant line was assessed from dominant peak on semi-logarithmic scale histogram (Fig. 1). The tetraploid plant detected in this cultured batch was obtained from treatment with 0.1 % colchicine, which was about 4.1 % (Table 1; Fig. 1). Consequently, two tetraploid plants (CC102 and CC110) showed the dominant peak at double channel (Fig. 1c, e) of that of diploid (WT1) plant (Fig. 1a). On the other hand, squash technique was used to determine the chromosome numbers at metaphase chromosomes. The wild type plant (WT1) showed normal diploid chromosome number as 2n = 2x = 28 (Fig. 1b) as reported previously (Kiehn 1996), while lines CC102 and CC110 obtained from 0.1 % colchicine were confirmed for their tetraploidy (2n = 4x = 56) as shown in Fig. 1d and f, respectively. This confirms the effect of colchicine that can inhibit microtubule polymerization which further effects on chromosome segregation leading to chromosome doubling. There are several factors involved in polyploidy induction by colchicine such as concentration, treatment period, explant type, and etc. For example, colchicine-induced tetraploid root tips of *Echinacea purpurea* were investigated at different treated time (24, 48, 72 h.) and gave 4–5 % induction efficiency depending on period of treatment (Abdoli et al. 2003). Similar result was reported by Gu et al. in 2005 when percentage of tetraploid production in *Zizyphus jujuba* Mill. cv. Zhanhua (1.7–5 %) was varied according to different

concentrations and exposure times of colchicine treatment. Consideration on the percentage of tetraploid induction in *M. hirtus* gained from our research that using in vitro shoot apices as donor explants (Table 1), about 4.1 % of tetraploid induction was not less than those pervious mentioned reports. However, several factors involved in experimental methodology were suggested to be investigated and optimized in future work in order to improve the efficiency of tetraploid induction with minimized plant death. Colchicine is frequently used for polyploid induction in several plants, but it also affects survival and regeneration ability of plants after treatment. Colchicine treatment with high concentration and long duration increased in mortality of shoot apex in *Gerbera jamesonii* Bolu cv. Sciella (Gantait et al. 2011). Low shoot regeneration of *Artemisia annua* L. was observed after treatment with high concentration of colchicine (Banyai et al. 2010). In this study, it was also suggested that colchicine concentration at 1 % seemed to be too high for shoot apex of *M. hirtus* since it caused 100 % plant death. Therefore, the concentrations in the range between 0.1 to 1 % colchicine should be further focused in order to obtain the appropriate concentration for high percentage of polyploidy induction in *M. hirtus*.

2.3.2 Growth characteristics of diploid and tetraploid plants

Morphological variations such as smaller or bigger leaves, lower plant height, larger stomata and a decrease in stomata density have been observed in many plants between diploid and polyploids induced after treatment with colchicine (Banyai et al. 2010; Gantait et al. 2011; Liu et al. 2011; Ye et al. 2010). In the present study, tetraploid induction using colchicine changed morphology of *M. hirtus* as observed in in vitro root growth, leaf size and guard cell character as shown in Fig. 2 and Table 2. In particular, both tetraploids CC102 and CC110 generated thicker roots than diploid plant WT1 (Fig. 2B). Among two tetraploids and one diploid, the smallest leaf length was observed in tetraploid CC110 (Fig. 2C; Table 2) while no difference in leaf width was observed among them. In this study, tetraploids CC102 and CC110 had larger size of guard cells in both width (1.3–1.5 times) and length (1.4–2.2 times) than diploid WT1, while the density

of guard cells in tetraploid plants especially in CC110 was around 1.7–4.1 times lower than that in the diploid (Fig. 2D; Table 2). After transplantation to the pot, plant height, number of shoots, number of leaves, fresh weight of upper parts, and fresh and dry weights of leaves were compared among diploid and tetraploid plants. Diploid (WT1) was taller than tetraploid (CC110) plant, while number of shoots and fresh weights of upper part and leaves of tetraploids were larger than those of the original diploid plant (Fig. 3; Table 3). Identification of differences between normal diploid and tetraploids gained from this study has been done in a number of ways, including cytological, morphological and anatomical characteristics. This has been referred to line characteristics and also to plant properties in previous reports. A number of studies have shown stomata characteristic (size and density) to be an effective criteria for polyploid identification (Cohen and Yao 1996; Gu et al. 2005; Mishra 1997). Two of our tetraploid lines showed larger stomata size (width-length) than diploid wild type. Line CC110 showed an increase in guard cell size (length and width) 2.2 and 1.5 times larger than WT1. Similar results for colchicine induced tetraploids on guard cell size has been demonstrated in *Echinacea purpurea* (Nilanthi et al. 2009), *Vicia villosa* roth (Tulay and Unal 2010) and *Artemisia annua* (Banyai et al. 2010). The size of guard cells directly correlates to the density (no. mm^{-2}) of guard cells in the plant leaf. In our experiment on *M. hirtus*, the largest sized guard cells found in tetraploid line CC110 showed the lowest guard cell density (42.4 no. mm^{-2}). This was lower than CC102 (2.49) and WT1 (4.19). The stomatal density of tetraploid *Vicia villosa* was also lower than those of diploid plant as reported by Tulay and Unal (2010). Generally, size and density are inversely correlated as observed in numerous plant species (Franks and Beerling 2009). This is due to stomatal pores, which are microscopic structures on the epidermis of leaves formed by 2 specialized guard cells that function involve to control the water evaporation and gas exchange (CO_2) between plants and the atmosphere. In neem tree (*Azadirachta indica*), it was found that the stomatal density was positively correlated with net photosynthesis (Kundu and Tigerstedt 1998). The stomatal size and density determine maximum stomatal conductance of CO_2 to sites of assimilation. The larger stomatal size might

promote photosynthetic capability, nevertheless may also cause increasing rate of transpiration according to the loss of water vapor to atmosphere. Therefore, the optimum stomatal size and density that could facilitate photosynthesis by not trigger overrate of plant water loss would be considered. The important anatomical features measured in terms of stomatal size and density are therefore directly relevant to stomatal conductance, which was coordinate with mesophyll photosynthesis and water vapor. These factors will further affect plant growth and development (Lawson 2008; Mansfield et al. 1990, Warner and Edwards 1993; Wong et al. 1979). In *M. hirtus* some parameters of the morphological growth (shoot number, aerial part FW) of tetraploid CC110 were highest. This may suggested that CC110 has better growth and development than CC102 and WT1, respectively. Morphological differences between diploid and tetraploid plants demonstrated in this study in terms of shoot height, number of lateral shoot, and fresh weight of upper part referred to potential of tetraploid to produce higher yield than diploid, especially in CC110. However, considering on the leaf part, which was the major source of plant extract, the tetraploid line CC102 gave highest FW and DW. Therefore, the difference of morphology among lines will be used as one of key parameters for selection of potent line according to yield.

2.3.3 Antibacterial activity and analysis of biochemical compounds in diploid and tetraploid plants

The antibacterial activity of *M. hirtus* crudes against Gram positive bacteria was performed using *S. aureus* and *B. subtilis* and determined by inhibition zone (mm) after 8, 16, 24 h after incubation. The result in Table 4 and Fig. 4 revealed that all methanolic crudes extracted from WT1, CC102 and CC110 showed bioactivity against a non-pathogenic bacterium, *B. subtilis*. The highest activity was observed in crude extracts of CC102, which was highly significant different from CC110 and WT1, respectively. Interestingly, the crude extracts of CC102 showed activity against *S. aureus* after 16 h of incubation, whereas those of WT1 and CC110 showed no activity. This is the first report demonstrating the potential of *M. hirtus* extract against pathogenic bacteria. Since *S.*

aureus is a virulent human pathogen which common cause infection in hospitalized patients by causing infections and syndromes such as impetigo bullosa, pyomyositis, surgical wound infection, pyomyositis, empyema, toxic shock syndrome, and foodborne gastroenteritis (Archer 1998; Corey 2009), the bioactivity against this bacteria detected from methanolic crudes of CC102 reveals the possibility of novel or abundant antimicrobial compounds produced in this tetraploid line. Line CC102 also shows potential for use as an alternative source of medicinal plants used for anti-infectious disease caused by *S. aureus*. The concentrations of tested crude extracts used in our study was 2 mg/disc, which was in the appropriate range compared to previous reports (Sule et al. 2011; Zaidan et al. 2005) that applied 1–2 mg/disc of crude extracts from leaves of *Andrographis paniculata*, methanolic extract of *Morinda citrifolia* and *Piper sarmentosum* to examine the growth inhibition of *S. aureus*. In addition, it would also be interesting to test the bioactive potential of *M. hirtus* against other Gram positive pathogenic bacteria with crudes extracts obtained from different solvents.

It is interesting to note that line CC102 shows properties unique to line CC110, as they are both induced tetraploids. Therefore, it was important to identify the biochemical variation present both quantitatively and qualitatively between the two tetraploid lines. The biochemical analysis by GC–MS of methanolic extracts detected from diploid WT1, tetraploid CC102 and CC110 was demonstrated in Fig. 5. Heat map visualization of the relative difference between methanolic extracts of diploid (WT1) and tetraploids (CC102 and CC110) of *M. hirtus* was analyzed by the HCA. The results showed that there are some candidate compounds detected only in CC102 that grouped in the same cluster of bioactivity against *S. aureus*, such as 9-Octadecyne (2); Stigmast-5-en-3-ol, oleate; 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-. The antioxidant and antimicrobial activity of 9-Octadecyne (2) had been reported (Upgade and Bhaskar 2013), while Stigmast-5-en- 3-ol, oleate compound showed the antihyperlipidemic and anti-tumor activities (Iyer and Patil 2012). The compound 9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)- presented its antibacterial, anti-inflammatory, and antioxidant properties as mentioned by Kalaivani et al. (2012). Besides the unique compounds found only in

CC102, it was also found that some compound for example 9,12,15-Octadecatrienoic acid, (Z,Z,Z)- and bicyclo[3.1.1]heptane, 2,6,6- trimethyl-, (1.alpha., 2.beta., 5.alpha.)- that could be detected in both CC102 and CC110 tetraploids but absent in diploid WT1, were reported to have antibacterial properties and anti-parasite (Lee et al. 2002; Sartorelli et al. 2012). The amounts of these compounds observed in CC102 were about 1.3–2 folds higher than CC110. Therefore, it is possible that the evidence of synergistic effects between antibacterial compounds found only in CC102 in combination with abundant accumulation of compounds in this tetraploid line could promote the bioactivity of CC102 against *S. aureus*.

In this present report, the effects of ploidy are presented in terms of plant morphology as well as the resulting bioactivities and biochemical constituents from leaf extracts of *M. hirtus*. The line-specific characteristic was notably observed even though among tetraploidy (CC102 and CC110). Although tetraploids that were induced in this experiment showed a doubling of chromosome numbers as 2n = 4x = 56, a mutation in karyotypic chromosome and genes may occur during abnormal process of cell division affected by colchicine treatment. Colchicine can induce numerous structural chromosomal aberrations in plant cells, which may further affect plant growth and development (Arni and Hertner 1997). Colchicine not only induces polyploidy of plant chromosome number but also causes cell abnormalities such as irregular nucleus shape and occurrence of micronuclei as observed in *Secale cereale* L. (Caperta et al. 2006). Thereby, the chromosome and DNA mutations in those abnormal nuclei may affect different gene expressions and cause variations in plant proteomes and metabolomes. Therefore, the difference in bioactivity between CC102 and CC110 is likely due to mutations occurring at the chromosomal and/or gene level rather than the genome level. The differences among tetraploid lines had been previously reported in *Artemisia annua* after colchicine treatment (Banyai et al. 2010) in terms of plant anatomy and terpenoid contents even though they were selected from the same batch of colchicine-inducing treatment. The line-specific tetraploids of *M. hirtus* observed in this present study (CC102 and CC110), therefore showed differences in plant morphology, bioactivity, and

biochemical constituents. Two novel anti-inflammatory saponins were detected in tetraploid plant of Jiaogulan (Gynostemma pentaphyllum) as demonstrated by Yang et al. (2013). Hence, we would like to communicate that the polyploid induction method is one of the alternative way to create the novel plant that have useful property as address here in case of tetraploid CC102 of *M. hirtus*. In order to reveal specific bioactive compounds found in the tetraploid line CC102 of *M. hirtus*, which showed antibacterial activity, methanolic extract should be purified and further analyzed. This would allow the identification of the primary compounds in CC102 that act against *S. aureus*.

2.4 Conclusion

This is the first report of the successful induction of tetraploids in *M. hirtus* using colchicine treatment. Tetraploid plants provided higher fresh weights of aerial parts and leaves. Moreover, bioactivity of tetraploid methanolic extract was higher than that of original diploid plants and one candidate tetraploid line CC102 showed activity against *S. aureus* which is a human pathogenic bacterium. Different bioactive properties for CC102 was addressed due to finding interesting compounds that could not be detected in WT1 and CC110 lines. These results suggest that the tetraploid lines of *M. hirtus* established in this study can be used as an important source for pharmaceutical application. However, several factors involved in the treatment methodology such as the type and concentration of the chromosome doubling agent, as well as treatment times should be investigated further. This would improve the efficiency of tetraploid induction and minimize plant death. Moreover, biochemical identification of the major bioactive compounds in crude extracts of line CC102 should be pursued further.

Table 1 Survival percentage of shoot apices after 1 month of colchicine treatment. The tetraploid induction of *Mitracarpus hirtus* L. was observed after 6 months of treatment.

Colchicine concentration (% w/v)	Number of treated shoots	Survival (%)	No. of plants obtained	
			Diploid	Tetraploid
0	20	100	20	-
0.01	38	100	38	-
0.10	49	100	47	2
1.00	10	0	-	-

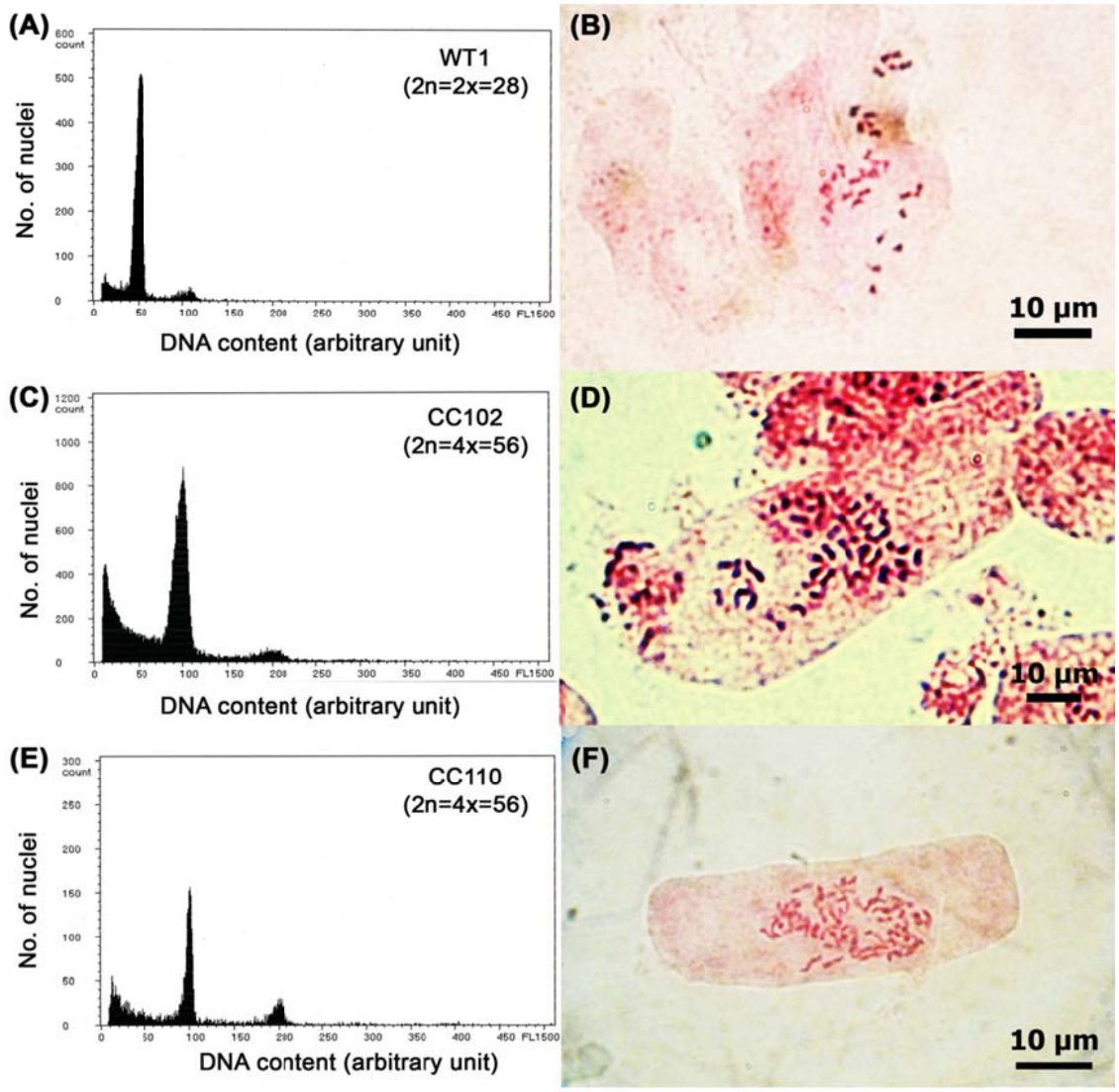

Figure 1 Flow cytometric histograms of leaf DNA contents and metaphase chromosomes in root tip cells of *Mitracarpus hirtus*. (A, B) diploid line WT1, (C, D) tetraploid line CC102, and (E, F) tetraploid line CC110.

Table 2 Comparisons of leaf and stomata characteristics of the diploid (WT1) and tetraploids (CC110, CC102) of *M. hirtus* cultured for 30 days in acclimatized conditions.

Code	Ploidy	Leaf length (cm)	Leaf width (cm)	Guard cell		
				Density (no./mm^2)	Length (μm)	Width (μm)
WT1	2x	3.9 ± 0.3 a	1.2 ± 0.3 ns	172.6 ± 27.5 a	30.3 ± 4.7 c	24.1 ± 4.4 c
CC102	4x	3.7 ± 0.5 a	1.0 ± 0.1 ns	100.1 ± 11.7 b	42.9 ± 4.4 b	30.9 ± 4.3 b
CC110	4x	3.1 ± 0.4 b	1.0 ± 0.1 ns	42.4 ± 6.7 c	67.8 ± 7.9 a	37.2 ± 5.7 a

Different letters within the column indicate significant difference of mean (±SD) tested by Duncan's Multiple Range Test (DMRT) at $p \leq 0.01$. The data were analyzed from 8 replications of each treatment.

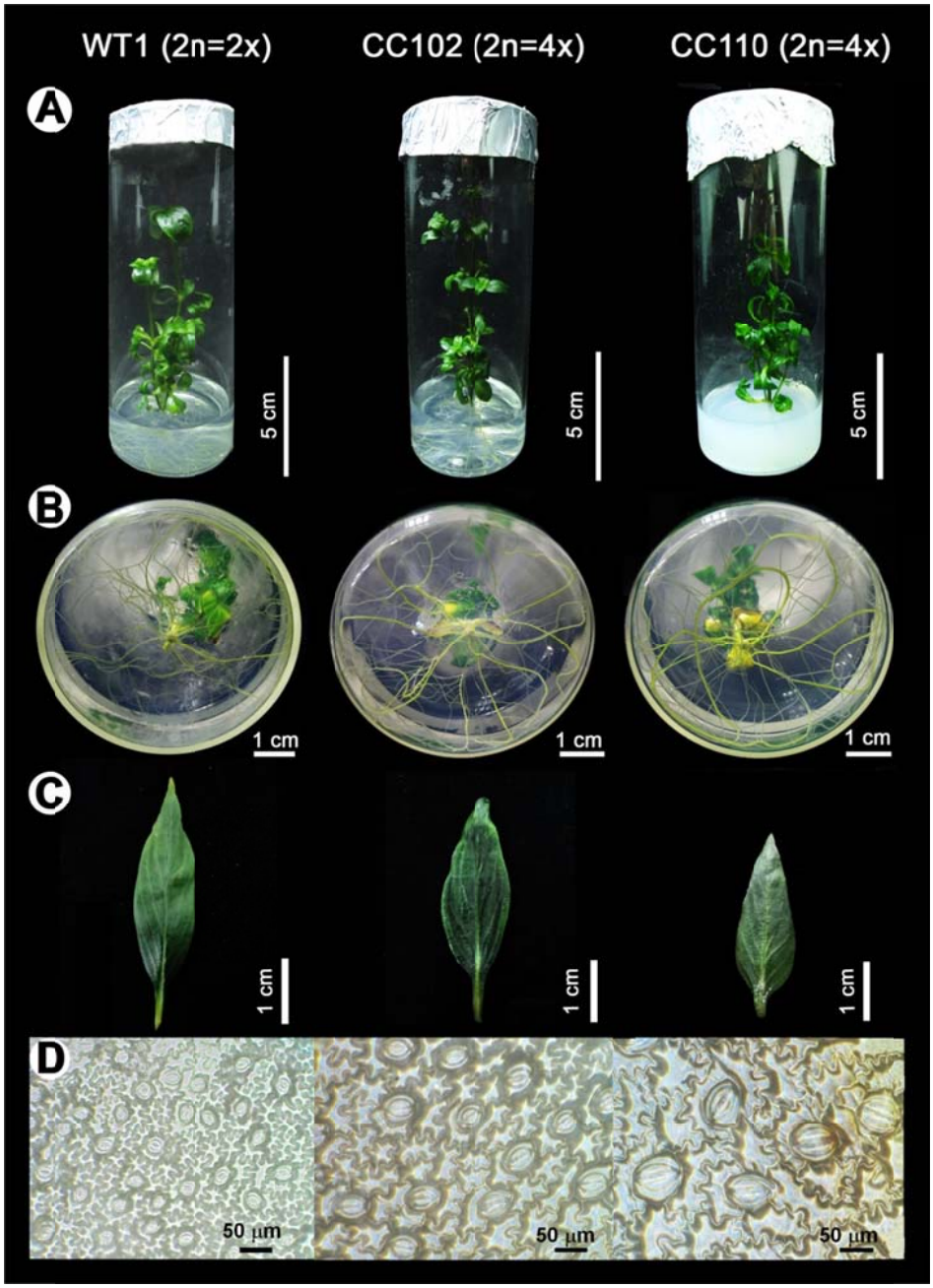

Figure 2 Comparison of morphological characters between diploid line (WT1) and two tetraploid lines (CC102 and CC110) of *Mitracarpus hirtus*. (A) plant height, (B) root growth, (C) leaf size, (D) stomata size and density.

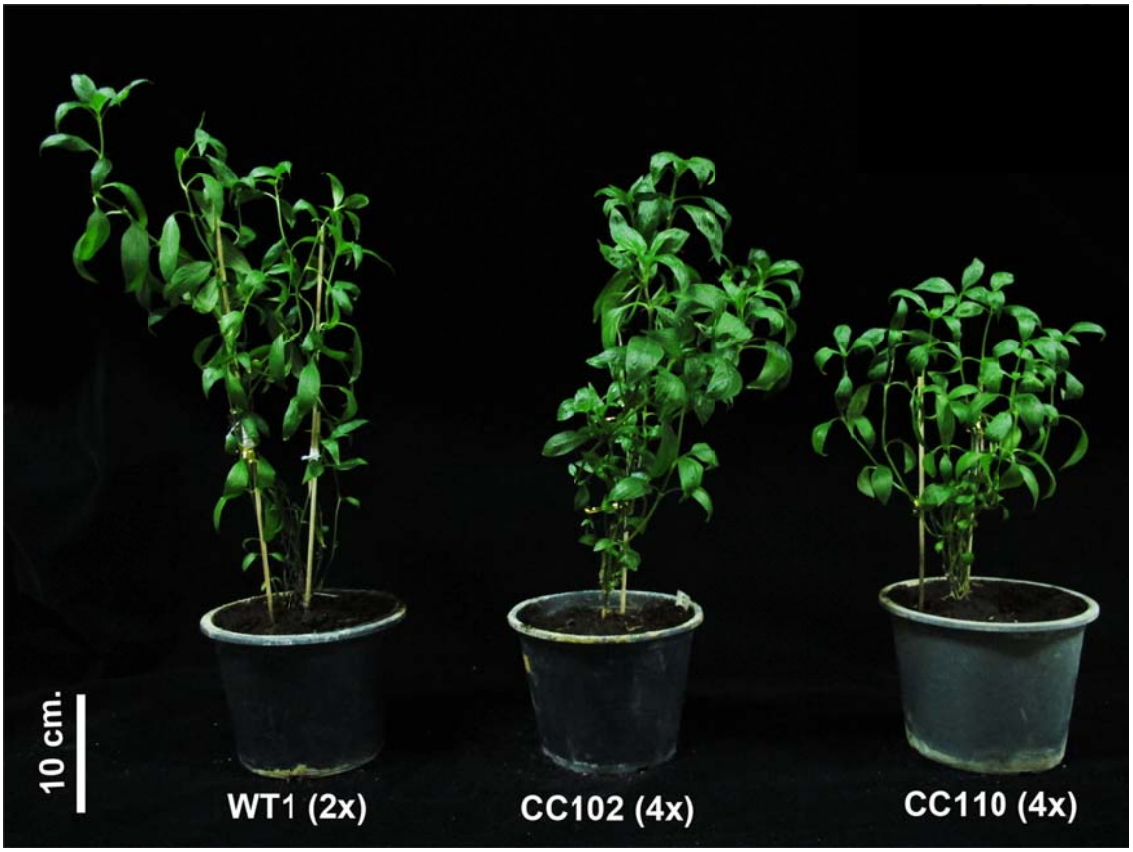

Figure 3 Diploid (WT1) and tetraploid (CC102 and CC110) plants 3 months after transplantation to pots.

Table 3 Growth characteristics of diploid (WT1) and tetraploid (CC102 and CC110) plants after 3 months of transplantation to pots (*ex vitro* conditions).

Code	Ploidy	Height (cm)	No. of shoot	Upper part FW (g)	No. of leaves	Leaf weight (g)	
						FW	DW
WT1	2x	39.6 ± 4.1	10.4 ± 2.8	11.3 ± 3.6	100.2 ± 38.6	5.1 ± 1.9	0.6 ± 0.2
CC102	4x	37.6 ± 4.4	12.9 ± 6.2	15.4 ± 5.6	73.6 ± 25.9	7.5 ± 2.2	0.8 ± 0.3
T-test		ns	ns	ns	ns	*	*
CC110	4x	34.4 ± 3.4	16.0 ± 5.6	16.8 ± 5.6	98.1 ± 29.9	7.0 ± 1.6	0.7 ± 0.2
T-test		**	**	*	ns	*	ns

The data (mean ±SD) were obtained from 10 plants each of tetraploid (CC102, CC110) and its original diploid (WT1) lines. Statistical analysis of significant difference among tetraploid (CC102 or CC110) and diploid (WT1) was analyzed by T-test.

* Significant difference at $p \leq 0.05$, ** highly significant difference at $p \leq 0.01$, ns: non-significant difference.

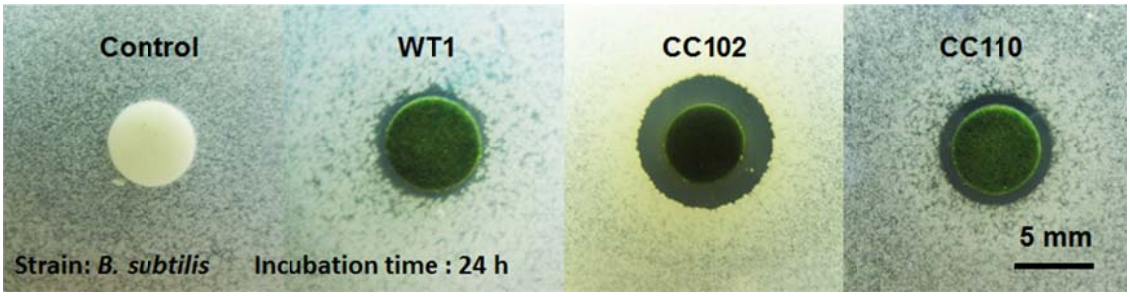

Figure 4 Effect of methanolic extract of *M. hirtus* against *B. subtilis*. Inhibition zone was observed at 24h after incubation extract absorbed-disc with *B. subtilis* in comparison between original diploid (WT1) and tetraploid (CC102 and CC110). DMSO 100% was used as control.

Table 4 Inhibition zone (mm) of diploid and tetraploid methanolic extract (2 mg/disc) of *M. hirtus* against 2 kinds of bacteria

Plant code	Ploidy	*S. aureus*			*B. subtilis*		
		Incubation time (h)			Incubation time (h)		
		8[1/]	16	24	8	16	24
WT1	2x	-	0.0 ± 0.0	0.0 ± 0.0	7.6 ± 0.2	6.9 ± 0.4	6.7 ± 0.2
CC102	4x	-	7.8 ± 0.4	7.2 ± 0.5	9.5 ± 0.7	9.3 ± 0.9	9.3 ± 0.9
T-test			**	**	**	**	**
CC110	4x	-	0.0 ± 0.0	0.0 ± 0.0	8.0 ± 0.5	7.5 ± 0.6	7.4 ± 0.5
T-test			ns	ns	ns	*	**

[1/] The inhibition zone was not clear because of slow growth of *S. aureus* at 8 hours

Diameter of disc was 6 mm. The data (mean ± SD) were obtained from 7 replications for each tetraploid (CC102 and CC110) and its original diploid (WT1) methanolic crude extract. Statistically significant difference between each tetraploid and diploid was analyzed with T-test.

* Significant difference at $p \leq 0.05$, ** highly significant difference at $p \leq 0.01$, ns: non-significant difference.

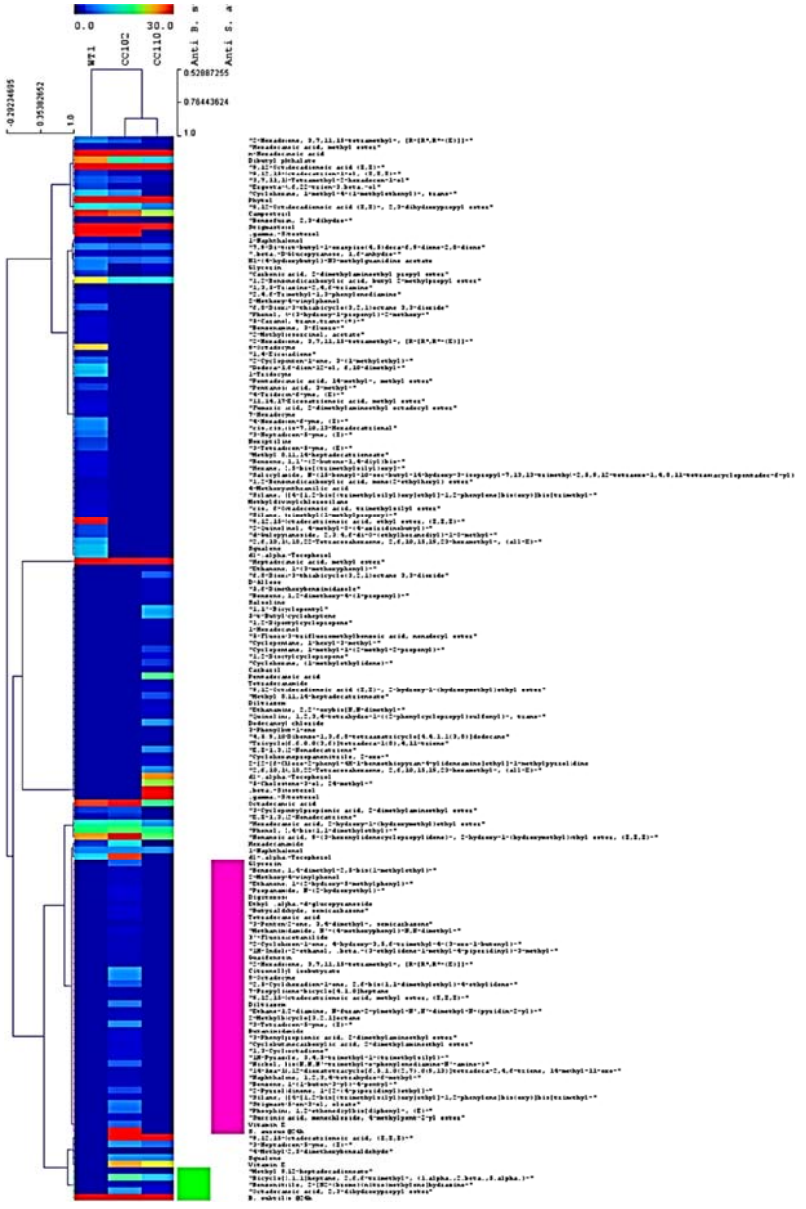

Figure 5 Heat map visualization of relative difference in methanolic extracts between diploid (WT1) and tetraploids (CC102 and CC110) of *M. hirtus* analyzed by the Hierarchical Clustering Analysis (HCA). The color scale ranged from blue (low content) to red (high content) as shown above the heat map. Vertical green indicated related compounds in anti-*B. subtilis* while vertical pink indicated the related compounds in anti *S. aureus* activity.

CHAPTER 3

Phytochemical alteration and new occurring compounds in hairy root cultures of *Mitracarpus hirtus* L. induced by phenylurea cytokinin (CPPU)

3.1 Introduction

Mitracarpus hirtus L., belongs to the Rubiaceae family, and is commonly distributed throughout gardens, farms and fields in tropical and neotropical regions. Common uses for this plant include use as an insecticide, antibiotic and antidote for insect stings and bites (Burkill 1970). Reports to date have focused on the primary phytochemical screening of the related species *Mitracarpus scaber* Zucc. and have revealed the presence of alkaloids, tannins, cardiac glycosides and saponins (Abere et al. 2007). Crude extracts of *M. scaber* have been shown both antibacterial and antifungal properties (Cimanga et al. 2004; Owolabi et al. 2013). Although there has no report on the active compounds of *M. hirtus*, the finding of bioactive compounds in *M. scaber*, and the medicinal qualities ascribed to *M. hirtus* indicates a possible likelihood of finding compounds of interest in *M hirtus*. Recently, there has a report that firstly demonstrated for biochemical and bioactive properties of *M. hirtus* from leaves extracts (Pansuksan et al. 2014). Since *M. hirtus* has its potential grow and distribute widely, it shows useful characters to provide accessible source of new alternative pharmaceuticals. However, additional biotechnological techniques used for commercial scale production is one of the significant factors that need to be considered. Commercial scale production of plant useful compound through cell and organ cultures for pharmaceutical industry requires several factors such as product safety, controllable over micro-environmental conditions (agitation, aeration, temperature, dissolved oxygen, pH, etc.), and low production cost. Screening of high-producing cell lines in combination with appropriate reactor system is generally required for establishment of secondary metabolite production. However, plant cell retains its

recalcitrant due to genetic instability, sensitive to reactor force, and generally has low or no accumulation of useful metabolite in the non-organized cell mass (Bourgaud et al. 2001). The hairy root has shown several advantages for industrial production of useful plant compounds over the cell system by incorporated with air-lift type reactor to solve the problem of sheer stress (Caspeta et al. 2005). Hairy root is characterized by ability to grow in plant growth regulator free-medium with a high growth rate, and biochemical and genetic stability (Srivastava and Srivastava 2007). This character is attractive for growth and proliferation in the reactor. Hairy root culture can be established using Ri plasmid of *Agrobacterium rhizogenes*, a soil born bacterium, which induces root formation by transfer and integrating of bacterial T-DNA (transfer-DNA) into the plant genome. Hairy roots have been established in many plants for secondary metabolite productions, for example, artemisinin and stigmasterol productions in hairy root of *Artemisia annua* (Xie et al. 2000).

Hairy root culture provides an effective tool for induction of novel metabolite that normally does not produce in intact plant, especially when combines with advanced technologies. For example, new sesquiterpene compounds produced in hairy root culture of *Artemisia annua* showed its property to inhibit tumor cell proliferation (Zhai et al. 2010a; Zhai et al. 2010b). The combined strategies applied to hairy root such as screening high-producing plant lines, modified culture conditions, addition of precursors, elicitation, biotransformation, etc. could improve yields of target compounds as reported (Kim et al. 2013; Satdive et al. 2007). Moreover, hairy root possibly secretes its metabolites to the medium, while cell system normally keeps in the vacuole. Elicitation is an attractive strategy due to its potential to enhance plant metabolite production and practical to apply using physical, biological or chemical elicitors. Various reports have demonstrated the application of elicitors on hairy roots that led to enhanced production of plant metabolites. For example, the hairy roots of *Artemisia annua* produced higher artemisinin content (an antimalarial compound) from 0.8 $mg.g^{-1}$ DW to 1 $mg.g^{-1}$ DW after treated with mycelial extracts from endophytic fungus *Colletotrichum sp.* (Wang et al.

2001). Among various types of elicitor, chemical elicitors using different kinds of stress-inducing compounds have been successfully applied to many plants due to their practicality and effectiveness. Several stress-inducing chemicals have been applied as elicitors to hairy root cultures. After treatments of 100 µM MeJ for 72 h or 200 µM SA for 96 h to the 30 day-old hairy root culture of soybean, total isoflavones productions were enhanced up to 10.7 and 5.8 folds, respectively however MeJ caused to reduce plant cell mass (Theboral et al. 2014). Therefore, finding of other alternative elicitors that are effective and lack the associated cell mass reduction needs to be considered. One of the key hormones controlling plant growth and development, cytokinins also share biosynthetic pathways with terpenoid compounds, a major plant secondary metabolite produced in plants. Cytokinin, therefore, plays an important role not only in plant growth and development but also in metabolite production. Therefore, alteration of cytokinin production either by internal interference in metabolic biosynthesis or by external application of abundant synthetic cytokinin to cultured plants may impact growth characteristics as well as secondary metabolites produced by treated plants. Previous reports have demonstrated that overexpression of isopentenyl transferase (*IPT*), a cytokinin biosynthetic gene increased both endogenous cytokinin (2-3 folds) and the sesquiterpene compound (artemisinin) up to 30-70% compared with the control (Sa et al. 2001). Cytokinin has been used as an elicitor to increase secondary metabolite production in several plants. Differences in cytokinin types and concentration were found to influence not only shoot proliferation, but also the *in vitro* production of bioactive secondary metabolites of *Aloe arborescens* Mill (Amoo et al. 2012). For exogenous treatment, a form of non-purine cytokinin is more effective and practical to use. The CPPU (2-chloro-4-pyridyl-N-phenylurea) is a non-purine based synthetic cytokinin which affects cell division, differentiation and plant development. In this study, the effect of CPPU on variation in plant secondary metabolite production in *M. hirtus* was demonstrated using the hairy root culture system. Since cytokinin generally promotes lateral shoot growth, the application of this synthetic CPPU on hairy root cultures may elicit root stress and lead to altered biochemical production in intact root tissue. As there

have been no reports on the hairy root induction of *Mitracarpus hirtus* L., this present study then demonstrates the first successful establishment of *M. hirtus* hairy root culture with a high proliferation rate. In addition, it is interesting to note that CPPU elicitation also altered overall phytochemical content and induced non-natural compound production in transgenic hairy root cultures of *M. hirtus.* After investigation by Gas Chromatography Mass Spectrometry (GC-MS) based metabolic profiling. Hairy root culture of *M. hirtus* established in this study provides an efficient system for secondary metabolite production, and may have pharmaceutical benefits through industrial production.

3.2 Materials and methods

3.2.1 Plant materials

Shoot apices of *Mitracarpus hirtus* L. (selected from Bangkok, Thailand) with 2-3 nodes were excised and surface sterilized with 0.5% sodium hypochlorite solution (Clorox®), they were rinsed 3 times with sterile distilled water prior to culture on 0.7% agar-solidified MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose and incubated under 25±2 °C, 60±5 %RH, illuminated daylight fluorescent lamp at 60±5 μmol.$m^{-2}s^{-1}$ PPF for 16 h photoperiod. Shoots were multiplied by subsequently subcultured *in vitro* shoot segments onto the same media composition every 30 days. The leaflets excised from *in vitro* plantlets were used as donor explants with *Agrobacterium rhizogenes* A13.

3.2.2 Hairy root induction by *Agrobacterium rhizogenes* A13 and proliferation of hairy root cultures

A stock culture of *Agrobacterium rhizogenes* A13 (Ohara et al. 2000) stored at -80 °C was selected and inoculated onto agar plates of 1.5% agar-solidified Luria Agar (LA) medium and incubated under 28 °C for 3-5 days. A single colony was then selected and used to inoculate a 125 ml flask containing 30 ml liquid Luria broth (LB) medium before

incubation for 16-18 h on a gyratory shaker (120 rpm) at 28 °C. Samples were then centrifuged at 3,000 rpm for 20 min. The bacterial solution was prepared by re-suspending cell pellets in fresh LB liquid medium. Leaflets of *Mitracarpus hirtus* were immersed in the bacterial solution; vacuum infiltration assisted transformation was then applied using an aspirator for 15 min followed by incubation on the shaker (120 rpm) for 5 min at 25±2 °C . The leaflets were collected and blot dried on sterile Whatman® paper before being placed on 0.7% agar-solidified MS medium containing 100 μM acetosyringone and incubated at 25±2 °C for 5 days. Control treatments were conducted by following the same protocol without bacterial inoculation. Leaf discs were then transferred to MS medium supplemented with 20 mg. L^{-1} meropenem in order to eliminate excess amounts of *Agrobacterium.* Leaf discs were retained in cultured media during subculture until *Agrobacterium* was found to be no longer present. The regenerated roots were then excised and transferred onto solidified PGR-free MS medium containing 3% (w/v) sucrose and routinely subcultured every 30 days for 5 months prior to PCR screen. The putative transformed hairy root was proliferated in the same formulation of MS liquid medium. To observe growth rate of hairy roots in comparison with non-transformed roots, 0.1 g of root fresh weight (FW) was transferred into each 125-ml Erlenmeyer flask containing 30 ml liquid MS media supplemented with 3% (w/v) sucrose. The cultures were incubated on a gyratory shaker (120 rpm) under 25±2 °C, 60±5 %RH. The experiments were cultured under two different conditions 1) with illuminated light at 60±5 μmol. $m^{-2}s^{-1}$ for 16 h. day^{-1} or 2) under dark condition for 24 h. The growth characteristics and root FW were recorded every four days for a total of 48 days. The total number of replications used for both hairy roots and non-transformed roots were 78 replications (one flask per one replication).

3.2.3 Confirmation of *rol* gene in hairy root

Plant genomic DNAs were isolated from non-transformed (negative control) and transformed hairy roots using a modified CTAB (cetylmethylammonium bromide)

method (modified from Doyle and Doyle 1990; Murray and Thompson 1980), whereas the extracted plasmid *A. rhizogenes* A13 was used as the positive control. PCR analysis was performed using a programmable DNA thermal cycler (Perkin Elmer, USA). The forward primer, 5'-CTGTACCTCTACGTCGACT-3' and reverse primer, 5'-TCAGTCGAGTGGGCTCCTTG-3' were used to amplify the 1.1 kb *rol* gene fragment (Kiyokawa et al. 1992). The final DNA amount was 0.25 µg/ 25 µl reactions using green GoTaq™. The PCR reaction was run with the following thermal cycling condition: 30 cycles 1 min at 93 °C, 1 min at 55 °C and 2 min at 72 °C (modified from Akutsu et al. 2004). A five microliter solution of PCR products were electrophoresed in 1 % (w/v) agarose gel at 100 voltages. Running gel was stained with ethidium bromide (0.5 µg. μl^{-1}) and visualized under UV light (Gel doc^{TM}). Hairy roots lines identified as PCR positive for the *rol* gene were selected, with integration of the *rol* gene into the genomic DNA confirmed by Southern blot hybridization. For Southern blotting, the isolated DNA (200 µg) was digested with *Eco*RI (BioLab, New England) at 37 °C for 20 h (Hosokawa et al. 1997; Ohara et al. 2000). The digested DNA was separated in 1 % (w/v) agarose gel by electrophoresis and transferred onto Hybond N^{+}® membrane (Amersham Biosciences, UK). A labeled 1.1 kbp of *rol* probe was prepared according to the manufacturer's instructions (Roche®, Germany). Hybridization of the probe with the membrane was carried out overnight at 68 °C. Hybridization signals were detected on membranes using the dye-generating redox reaction (NBT/BCIP Ready-to-Use Tablets, Roche).

3.2.4 CPPU treatment and analysis of biochemical compounds in hairy root culture of *M. hirtus*

0.1 g FW of hairy roots grown in liquid culture were excised and transferred into 125 ml size-flask with 30 ml liquid MS medium for total 10 flasks. Flasks were incubated on a shaker (120 rpm) under dark condition at 25±2 °C for 24 days (exponential stage). The hairy roots were treated with 0 (control) or 5 mg. L^{-1} CPPU (Kyowa Hakko Bio, Japan) for 48 hours. Consequently, the hairy roots were collected and

dried overnight at 50°C and the dried samples were ground with liquid nitrogen before extraction with methanol. Methanolic extracts were filtrated through Whatman® No.1 filter paper before evaporation of the solvent in an aeration hood. Crude extracts were re-dissolved with methanol to get the final concentration at 10 $mg.ml^{-1}$. The chemical constituents in crude extracts were analyzed by Gas Chromatography Mass Spectrometry (GC-MS)-based metabolite profiling. The final concentration at 10 $mg.ml^{-1}$ of each crude sample was analyzed using GC-MS with HP5-MS capillary using Agilent Technologies (USA) fused silica column 30 m × 0.25 mm × 0.25 µm (length×I.D.×film thickness). The oven temperature system was initially started at 50 ºC, increasing 10 $ºC.min^{-1}$ to 280 ºC. The temperature of GC-MS interface was 280 ºC. The injections of the extract were run at 1 ml. min^{-1} flow rate with 50:1 split ratio and mass range from m/z 35–600 (modified from Kalaivani et al. 2012; Owolabi et al. 2013; Roy et al. 2010). Heptadecanoic acid, methyl ester (C17) was used as an internal standard. The detected compounds were searched base on the NIST-08 (National Institute of Standards and Technology) library.

3.3 Results and discussion

3.3.1 Hairy root induction of *M. hirtus* using *Agrobacterium rhizogenes* A13 and confirmation of *rol* gene expression in hairy root

In this study, *in vitro* leaf-disc cultures of *M. hirtus* were successfully provided as potential donor sources of plant material for transformation with *A. rhizogenes* A13. After cultivation for 2-3 weeks the leaf-derived root regeneration was observed from both transformed and non-transformed cultures. Proliferation of adventitious roots in transformed culture showed a substantial increase during the subsequent cultures in solidified PGR-free MS media either with (Fig. 6A) or without (Fig. 6B) the original leaf tissues, whereas non-transformed roots had poor growth and proliferation in this media condition. Leaf-derived roots from non-transformed leaves died after maintenance in PGR-free medium for 60 days. The putative transformants were confirmed by PCR analysis, with the PCR-positive cultures for the *rol* gene (Fig. 6C). After subsequently

subcultured every 30 days for 5 months, putative transformants were confirmed for *rol* gene integration by Southern blot hybridization. It was found that the integration of *rol* in *M. hirtus* was observed in all PCR-positive root lines (Fig. 6D). Utilizing a root-inducing (Ri) plasmid, three transgenic lines (R106-62, R107-3, and R107-4) of *M. hirtus* with hairy root expression were obtained using *A. rhizogenes* A13-mediated transformation, a first for *M. hirtus*. The Ri-plasmid contained *rolA, B, C*, and *D* genes which are responsible for root formation in transformed cell. It was noted that different *A. rhizogenes* strains produce different types of opines, such as agropine, mannopine, cucumopine and mikimopine (Pal et al. 2013). These differences may cause interference in both the amount of hairy root production by *rol* gene, as well as potentially affecting the biosynthesis of plant secondary metabolites after transgene insertion. In our study, a wild strain of *A. rhizogenes* A13 (MAFF-02–10266; Daimon et al. 1990) was used and its Ri plasmid will direct the plant cells to produce Mikimopine (Hoshino and Mii 1998; Ohara et al. 2000).

3.3.2 Growth and proliferation of hairy root cultures

Transgenic hairy roots generally have a higher growth rate than normal roots as previously described in many studies. In our study in *M. hirtus,* although percentages of root induction from both the control (46.7 %) and transformed (45.3 %) leaves were not significantly different (data not shown), the transformed hairy root was obviously distinguished for its potential to proliferate on both solidified and liquid media. Comparison of growth and proliferation between transformed hairy roots of *M. hirtus* (line R107-3) with control roots (WT1, excised from *in vitro* plant) were investigated using liquid PGR-free MS medium (Fig. 7A). When comparing the root FW of R107-3 with the control WT1, an increase in hairy root production in R107-3 was found to be 3.8 times higher in lighted conditions (60±5 $\mu mol.m^{-2}.s^{-1}$); and 5 times greater in dark conditions. There had no significant difference between FW of WT1 roots cultured under light or dark conditions, similar result was also observed in those of R107-3 hairy root.

However, the growth characteristic of the root cultured under light showed the occurrence of shoot formation as demonstrated in Fig. 7B (WT1) and 7D (R107-3 hairy root). Normally, light affects several physiological responses including, seedling development, and vegetative development (Chen et al. 2004; Saitou et al. 1992). Approximately 12 times of shoot formation was increased when dim light was applied (17 $\mu.mol.m^{-2}s^{-1}$) to root culture of horseradish (*Armoracia rusticana*). The hairy roots of horseradish had a 3-4 times the shoot formation versus non-transformed roots in both light and dark conditions (Saitou et al. 1992). Interestingly, the shoot formation of *M. hirtus* (numbers and development) in WT1 was higher than R107-3 cultures (data not shown). Since our media condition contained neither cytokinin nor other PGRs, this could be determined for high shoot regeneration capability of *M. hirtus* induced by light in the culture system, even though transgenic hairy root performed lower shoot regeneration ability than control root. Hence, it is suggested that cultural conditions are recommended to apply for large-scale hairy root production is dark condition (Fig. 7E), whereas transgenic shoot derived from hairy root could be induced when applied light to the system (Fig. 7D).

3.3.3 Effect of CPPU on biochemical constituents of *M. hirtus* hairy root

The effect of CPPU, a synthetic cytokinin on biochemical variations in hairy root of *M. hirtus* was determined after 48 h of application. The array of phytochemical compounds of hairy roots treated with 5 $mg.L^{-1}$ CPPU were analyzed by GC-MS and compared with untreated samples. The detected compounds in methanolic extracts of hairy root were searched base on the NIST-08 (National Institute of Standards and Technology) library and identified 44 newly present, 47 absent, 28 with increased contents, and 15 with decreased levels for CPPU-treated roots compared to non-treated hairy roots. The compounds with qualities higher than 80% were then selected for further analysis. For the CPPU treatment we identified 6 novel compounds, 5 absent, 11with increased, and 5 with decreased amounts when compared with the control (Table 5, Table 6). This evidence clearly demonstrates the effectiveness of CPPU as a chemical elicitor

and its ability to induce biochemical changes in hairy root culture of *M. hirtus*. Among the 5 novel compounds, only one alkaloid, identified as Eseroline, 7-bromo-, methylcarbamate (ester), was observed. In addition, two phenolic compounds that increased in the CPPU-treated sample, chrysophanol (2.23 times) and 2-methoxy-4-vinylphenol (1.95 times) were reported to have bioactive properties. Chrysophanol, an anthraquinone derivative, inhibited cell growth in Hep3B liver cancer cells by cell death induction (Ni et al 2012) therefore it showed potent antitumor activity. Increasing content of 2-methoxy-4-vinylphenol (1.95 times) in CPPU-treated cultures may have higher bioactivity as antioxidant and antibacterial compounds more than non-treated roots. Numerous reports have shown that 2-methoxy-4-vinylphenol extracts from various plant species are mentioned to have antimicrobial and antioxidant activities (Gao et al. 2011; Silici and Kutluca 2005). In our previous study, methanolic extracts of diploid and tetraploid *M. hirtus* leaves showed activity against a human pathogenic bacterium, *Staphylococcus aureus* (Pansuksan et al. 2014). This provides an increased possibility to modify *M. hirtus* as an alternative source of pharmaceutical compounds either from leaves or hairy root culture, especially when combined with elicitation such as CPPU.

Generally, cytokinin is a plant growth regulator which functions in physiological and developmental processes including, promoting cell division, cell differentiation, axillary bud growth, response to biotic and abiotic stress, and leaf senescence (Mok and Mok 2001; Sakakibara 2006). Cytokinin is synthesized at various sites in the plant (Sakakibara 2006) however, the root tip is the major site of natural cytokinin biosynthesis (Emery et al. 2000). Cytokinins naturally accumulate in the root tip, shoot apical meristem, cambium, and immature seeds, then acts at various sites throughout the plant. The co-action of the hormones auxin and cytokinin work to maintain the root meristem size and growth in of the plant. As observed in *Arabidopsis thaliana*, auxin promotes cell division to sustain the root meristem activity while cytokinin promotes cell differentiation by repression of auxin signals and transportation (Moubayidin et al 2009). Cytokinin generally influences lateral root formation at an early

stage and also prevents the effects of auxin on root growth by interfering with cell division and pattern formation. Cytokinin and auxin control the meristem activities with an antagonistic action. Exogenous cytokinin N^6 Benzyl amino purine (BAP) inhibited primary root elongation and lateral root formation (Chang et al. 2013). The hairy root of *M. hirtus* driven by *rol* gene expression produced increasing endogenous auxin in root tissue. Therefore, exogenous application of cytokinin (CPPU) to hairy roots caused an excess abundance of cytokinin that might act antagonistically towards any auxin present and further trigger stress in hairy root growth. The stress thereby could be elicited by CPPU and result in the alteration of phytochemical expression. Plant secondary metabolites generally are classified into three major groups based on biosynthetic origins: terpenoids, nitrogen-containing alkaloids, and phenolic compounds (Crozier et al. 2006). The CPPU-treated hairy root induced the changes in plant metabolites (present, absent, increased, and decreased compounds). Possible quantitative or qualitative changes of plant metabolite will influence not only physiological growth & development, but also bioactive properties. Terpenoids are the most diverse class of secondary metabolites. They play a role in cellular function and maintenance, environmental interactions, plant–plant communication, and plant–insect/animal interactions (Pichersky and Gershenzon, 2002). Terpenoids have been used as pharmaceutical agents, flavoring agents, and perfumes (Zhang and Demain 2005). Alkaloids play a defensive role against insects and pathogens, and are used as stimulants, pharmaceuticals, narcotics and poisons (Wink 1998). From this study, identified compounds with qualities higher than 80% presented in Table 6 could be grouped and identified as phenols, alkaloids, terpenes, fatty acids, and others. The results indicated significant changes in metabolic content, including four phenolic increases (i.e. 9,10-Anthracenedione, 1,8-dihydroxy-3-methyl-; 2-Methoxy-4-vinylphenol; and 4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol) and one novel alkaloid present (Eseroline, 7-bromo-, methylcarbamate(ester)) after elicitation with CPPU.

In order to describe the trend of metabolite flux altered after CPPU elicitation, the possible biosynthetic diagram of related compounds detected and identified in this study was shown in Figure 8. Three major biosynthetic pathways which were shikimatic, cytosol MVA, and plastidial DXP/MEP pathway, were mentioned in this study. Cytokinin can be biosynthesized through dimethylallyl diphosphate (DMAPP) from the plastid. Application of exogenous cytokinin (CPPU) has the possibility to effect endogenous cytokinin production and accumulation resulting in feedback of other metabolites production in related pathways. The abundant content of cytokinin due to CPPU, plus those naturally produced by the plant may result in altering the metabolic pathway in *Mitracarpus hirtus* hairy root. Increasing contents of two phenolic compounds, chrysophanol and 2-methoxy-4-vinylphenol were observed with CPPU application. Chrysophanol or 9, 10-Anthracenedione, 1,8-dihydroxy-3-methyl- (Emam and Abd El-Moaty 2009) was increased up to 2.23 times after CPPU elicitation. Chrysophanol was biosynthesized from isopentenyl diphosphate (IPP) which is derived from the MVA pathway, conversion of DMAPP, or 1-Hydroxy-2-methyl-2-(E)-butenyl 4- diphosphate (HMBPP) from MEP pathway. Another compound, 2-methoxy-4-vinylphenol (*p*-vinylguaianol) was synthesized from the shikimate pathway by conversion of ferulic acid and was observed to have increased after CPPU treatment (1.95 times). It is interesting to note that an alkaloid compound, Eseroline, 7-bromo-, methylcarbamate(ester) was identified in CPPU-treated hairy root. This compound (2.27 % content) could not be detected in non-treated samples. This alkaloid was synthesized from tryptophan derived from chorismic acid in the shikimate pathway. Therefore, it has been shown that CPPU application could regulate the metabolite flux, which has an effect on phytochemical alterations in *M. hirtus* hairy root. However, the types and concentrations of other elicitor should be considered for better production of target compounds. Methyl jasmonate (MeJ), a volatile organic compound also shows potential for use as a chemical elicitor. It was reported to stimulate secondary metabolites in many plants. Therefore, it may suggest application of MeJ to *M. hirtus* hairy roots in further experiments.

3.4 Conclusion

In summary, the successful establishment of transgenic hairy root of *M. hirtus* by *A. rhizogenes* A13 has been accomplished for the first time as reported in this study. Moreover, hairy root lines developed from *M. hirtus* have shown high potential for transgenic shoot production with *rol* gene expression. The information gained from this study indicates that *M. hirtus* hairy root shows potential as a model system for secondary metabolite productions in the bio-reactor due to its high proliferation, and could provide an alternative source of pharmaceutical compounds, and could be successfully used to induce new and potentially useful compounds after elicitation.

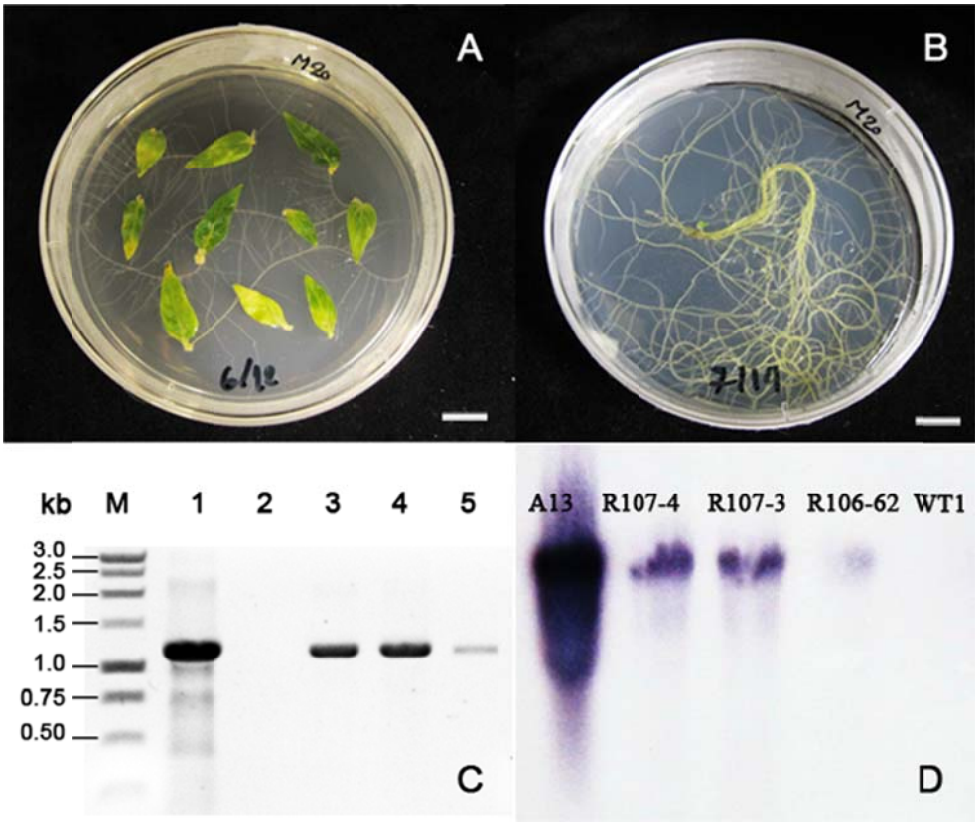

Figure 6 Hairy root formation after co-cultivated leaflet explants of *Mitracarpus hirtus* L. with *Agrobacterium rhizogenes* A13 for 3 weeks (A). Hairy roots were detached from explants and subsequently proliferated on solidified PGR-free MS medium with 1 month-subcultured intervals (B). PCR amplification of *rol* gene, lane M: marker, lane1: positive control of extracted plasmid *A. rhizogenes* A13, lane 2: negative control of non-transformed root (WT1), lane 3-5: DNA from hairy root lines (C).Southern blot hybridization of PCR-positive transgenic lines, the genomic DNAs of transgenic hairy roots (lines R107-4, R107-3, R106-62) digested with *Eco*RI performed positive band detected with *rol* probe (D). Bar = 1 cm.

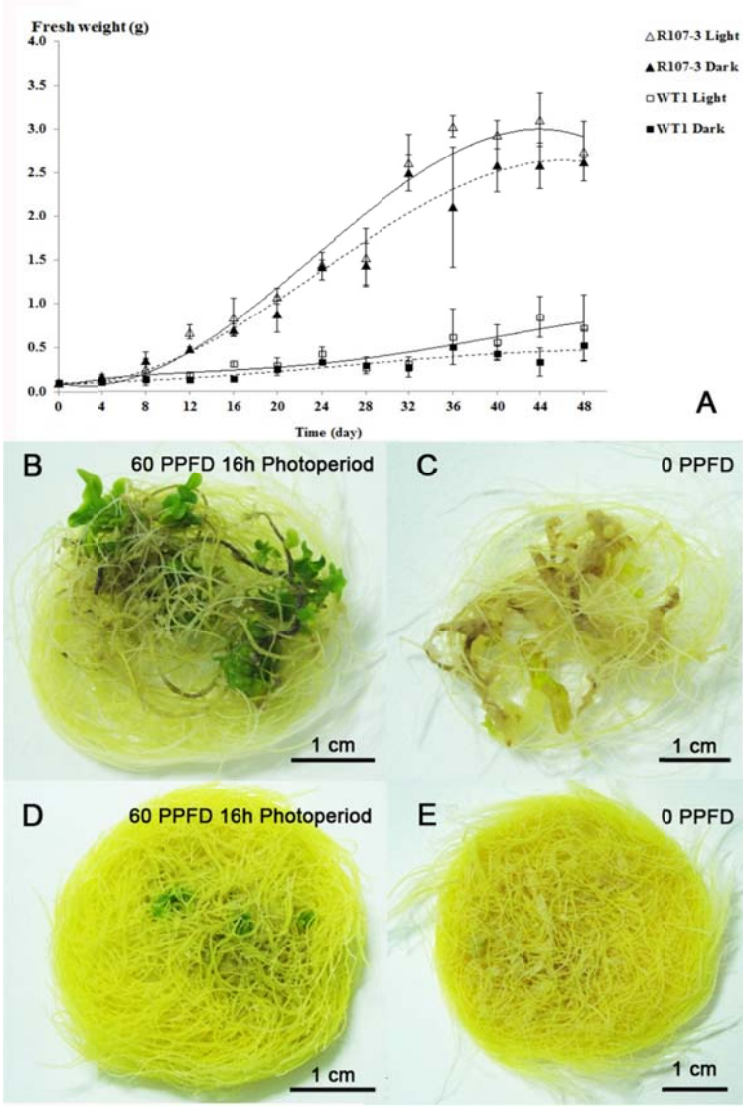

Figure 7 Comparison of growth and proliferation efficiency between non-transformed root (WT1) and transformed hairy root (lineR107-3) of *Mitracarpus hirtus* L. (A) Fresh weight (g) of hairy root, R107-3 and non-transformed WT1cultured in PGR-free MS liquid medium and incubated under light, with a 16 h photoperiod (illuminated with fluorescent lamp at 60 ± 5 µmol $m^{-2}s^{-1}$ PPF) or dark conditions (0 PPF, 24 h). Growth and proliferation characteristics of WT1 roots cultured under light (B) and dark (C) conditions. Hairy root line R107-3 cultured under light (D) and dark (E) conditions.

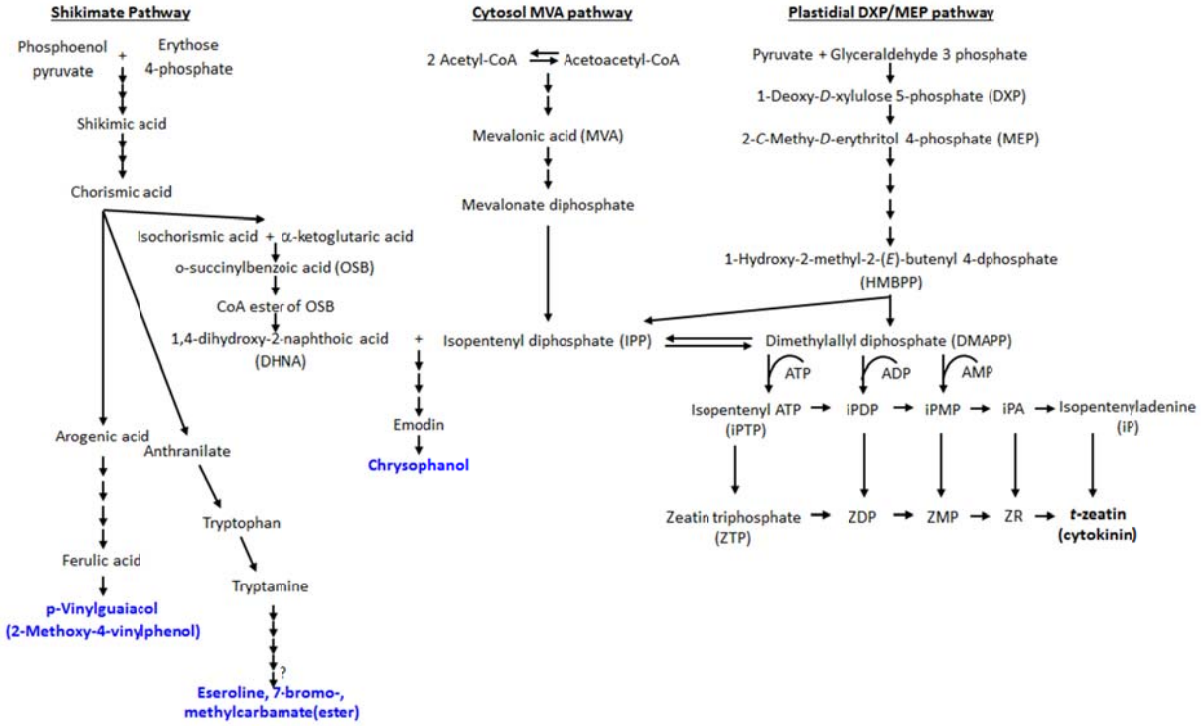

Figure 8 Biosynthetic diagram demonstrates for the possible relationship between the plastidial DXP/MEP, cytosol MVA, and shikimate pathways (modified from Anderson 1985; Farah and Donangelo 2006; Liu et al. 2005; Peleg et al. 1992; Quevedo et al. 2010; Tatsuo 2011) that are influenced by exogenous application of cytokinin (CPPU). The abundant content of cytokinin from CPPU plus those produced naturally may result in alterations of metabolic pathways in *Mitracarpus hirtus* hairy root. Increasing contents of two phenolic compounds, chrysophanol (2.23 times) and 2-methoxy-4-vinylphenol (1.95 times) were observed after CPPU application. An alkaloid compound, Eseroline, 7-bromo-, methylcarbamate (ester) was identified in post CPPU-treated hairy root. This compound could not be detected in non-treated samples.

Table 5 The GC-MS identification of biochemical constituents from methanolic extracts of *Mitracarpus hirtus* hairy root with or without 5 $mg.L^{-1}$ CPPU treatment. The compounds blasted with NIST08 library were normalized with internal standard (heptadecanoic acid, methyl ester).

RT (min)	Compound	Qual [a/]		Compound content (%)	
		Cont.	CPPU	Cont.	CPPU
4.83	1,2-Cyclopentanedione	91	91	9.79	16.00
6.80	2-Propanamine, N-methyl-N-nitroso-	80	64	1.33	2.55
7.51	2-Furancarboxaldehyde, 5-(hydroxymethyl)-	87	83	5.56	5.21
8.20	2-Methoxy-4-vinylphenol	83	91	3.02	5.90
9.23	Chloroacetic acid, dodecyl ester	-	91	-	5.95
9.23	Cyclododecane	93	-	7.01	-
9.53	Phenol, 2,4-bis(1,1-dimethylethyl)-	94	95	13.67	16.97
11.32	4-((1E)-3-Hydroxy-1-propenyl)-2-methoxyphenol	80	94	9.73	10.74
12.38	E-14-Hexadecenal	93	96	1.94	3.61
12.84	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	98	99	3.04	4.28
13.02	n-Hexadecanoic acid	98	99	16.75	12.87
13.22	Eseroline, 7-bromo-, methylcarbamate (ester)	-	92	-	2.27
13.61	Heptadecanoic acid, methyl ester	99	99	100.00	100.00
14.10	Cyclohexadecane	-	91	-	1.48
14.51	Methyl 9,12-heptadecadienoate	-	83	-	10.76
14.52	9,12-Octadecadienoic acid (Z,Z)-	98	96	12.92	10.92
14.57	Methyl 8,11,14-heptadecatrienoate	70	86	13.71	10.62
14.72	Octadecanoic acid	98	58	3.92	5.22
14.73	Pentadecanoic acid	91	-	4.11	-

RT (min)	Compound	Qual [a/]		Compound content (%)	
		Cont.	CPPU	Cont.	CPPU
17.65	1-Hydroxy-4-methylanthraquinone	76	89	1.76	3.87
17.72	Octadecanal	80	-	3.03	-
17.73	Oxirane, heptadecyl-	87	-	2.02	-
18.32	9,10-Anthracenedione, 1,8-dihydroxy-3-methyl-	81	81	43.63	97.22
18.95	E,Z-1,3,12-Nonadecatriene	83	89	3.85	4.39
25.99	Ergost-7-en-3-ol, (3.beta.)-	-	87	-	9.02
25.99	Campesterol	99	-	10.15	-
26.66	Stigmasterol	99	90	18.49	14.43
27.95	.beta.-Sitosterol	95	89	8.04	4.62

[a/] Qual = Percentage of Quality when blast with NIST08 Library (National Institute of Standards and Technology). Compound contents (%) presented in this table were selected only compounds with qualities higher than 80%.

Table 6 Grouping of compounds with qualities (Qual) higher than 80% presented after treated *M. hirtus* hairy root with 5 $mg.L^{-1}$ CPPU and analyzed by GC-MS.

No.	Type of compounds	Presence	Absence	Increase	Decrease
1	Phenols, Polyphenols and derivatives	-	-	4	-
2	Alkaloids	1	-	-	-
3	Terpenes	2	1	-	2
4	Fatty Acids and Conjugates	1	2	2	2
5	Others	2	2	5	1
	Total	**6**	**5**	**11**	**5**

Chapter 4
General discussion and conclusion

From this study, clone improvement of *Mitracarpus hirtus* was successfully established using biotechnological tools. Polyploid induction of *M. hirtus* via colchicine treatment was described in chapter 2. After screening the plants treated with colchicine, two tetraploid (CC102 and CC110) with double of chromosome number (2n = 4x = 56) were obtained at colchicine concentration of 0.1% w/v. Colchicine treatment with high concentration and long duration increased in mortality (Gantait et al. 2011) and low shoot regeneration in plant (Banyai et al. 2010). Since the 1% w/v of colchicine caused 100% death of *M. hirtus*, the concentration in range between 0.1 to 1 % colchicine should be further focused in order to obtain the suitable concentration for high percentage of polyploid induction. Different plant characteristics between diploid and polyploid were mentioned in many reports (Banyai et al. 2010; Cohen and Yao 1996; Gu et al. 2005; Mishra 1997; Nilanthi et al. 2009; Tulay and Unal 2010). Anatomical and morphological differences between diploid and tertraploids of *M. hirtus* were observed. Espectially in consideration of the leaf part, the major source of plant extract, the tetraploid line CC102 gave the highest FW and DW. Therefore, the fresh and dry weight of plants will be used as a key factor for selection of potential line-giving the high yield. The other important parameter for clone selection in pharmaceutical aspect is bioactivity. From the results, all methanolic crudes extracted from WT1, CC102 and CC110 showed bioactivity against *B. subtilis* and only CC102 extract inhibited the growth of *S. aureus*. Consequently, biochemical analysis by GC–MS and HCA showed some candidate compounds which were detected only in CC102 and grouped in the same cluster of bioactivity against *S. aureus*. Besides of those compounds, other interesting metabolites were observed in both CC102 and CC110 tetraploids but absent in diploid WT1. Those compounds were reported as antibacterial, anti-inflammatory, and antioxidant agents (Kalaivani et al. 2012; Lee et al. 2002; Sartorelli et al. 2012). Therefore, it is possible that antibacterial

compounds found only in CC102 in combination with abundant accumulation of others in this tetraploid line could synergistically promote the bioactivity of CC102 against *S. aureus*. In the present report, the line-specific characteristic was notably observed even among tetraploids (CC102 and CC110). This might have occurred as the results of cell abnormalities induced by colchicine as reported by Caperta et al. in 2006, leading to different gene expressions and further affecting variations in plant proteomes and metabolomes. Therefore, the difference in bioactivity between CC102 and CC110 is likely due to mutations occurring at the chromosomal and/or gene level rather than the genome level.

In order to produce hairy root for further industrial application, *Agrobacterium rhizogenes*-mediated transformation method was studied and presented in Chapter 3. Transgenic hairy root induction of *M. hirtus* with higher mass production when compared with non-transgenic one was achieved using wild strain of *A. rhizogenes* A13. Hairy roots containing *rol* gene were elicited by 5 mg L^{-1} CPPU and the detected compounds with quality that was higher than 80% after blast with NIST-08 library showed 6 presences, 5 absences, 11 increases, and 5 decreases differed from the control. Among 5 new presenting compounds, only one identified alkaloid was observed. On the other hand, two increasing phenolic compounds in CPPU-treated sample were reported to have bioactive properties. In our previous report, methanolic extracts of diploid and tetraploid *M. hirtus* leaves showed antibacterial activity (Pansuksan et al. 2014). This provided possibility to modify *M. hirtus* as an alternative source of pharmaceutical compound either from leaves or hairy root culture, especially when combined with elicitation with such as CPPU. The stress elicited by CPPU resulted to the alteration of metabolite production in hairy roots. The results showed the outstanding trend as follows: there were increases of four phenols (i.e. 9,10-anthracenedione, 1,8-dihydroxy-3-methyl-; 2-methoxy-4-vinylphenol; and 4-((1E)-3-hydroxy-1-propenyl)-2-methoxyphenol) and presence of one alkaloid (eseroline, 7-bromo-, methylcarbamate(ester)) after elicitation. These results implied that CPPU application could regulate the metabolite flux, which affected phytochemical alterations in *M. hirtus* hairy root.

From all of the results obtained from this study, the outstanding matters were concluded as follows;

1. Colchicine is a useful tool for polyploid induction in *M. hirtus* at the concentration of 0.1% w/v using excised shoot as target material.

2. Double number of chromosome set affect phytochemical production in tetraploids of *M. hirtus* and resulted in increasing of antibacterial activity against *S. aureus* growth.

3. *Agrobacterium rhizogenes* A13-mediated transformation was proved to be effective method for hairy root production of *M. hirtus.*

4. Elicitation by CPPU affected metabolite flux in hairy root culture of *M. hirtus.*

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SUMMARY

Biotechnology plays an important role in clone improvement. In present study, polyploidy induction using colchicine treatment and transformation via *Agrobacterium rhizogenes* were applied to *M. hirtus*. It was interesting to produce tetraploid plant with increasing the bioactive metabolites to this plant. Moreover, hairy root, the differentiated culture was also effective system for metabolites production in industrial application. Not only internal factors which affected the metabolite production in plant, but the external factors such as environment or stress also influence on plant behavior. Therefore, elicitation using CPPU was applied to *M. hirtus*. In this study, plant biotechnology was used to enhance efficiency of *M. hirtus* in order to serve as a new chance for pharmaceutical or other application in the future.

Chapter 2: Tetraploid induction of *Mitracarpus hirtus* L. by colchicine and its characterization including antibacterial activity

This is the first successful of tetraploids induction in *M. hirtus* using colchicine treatment. Tetraploid plants (CC102 and CC110) with chromosome number as 2n = 4x = 56 provided higher fresh weights of aerial parts and leaves, moreover, bioactivity of tetraploid methanolic extract was higher than that of original diploid plants. The potential candidate tetraploid line CC102 showed activity against *S. aureus*, a human pathogenic bacterium, due to the finding interesting compounds that could not be detected in WT1 and CC110 lines. It suggests that the tetraploid lines of *M. hirtus* can be used as an important source for pharmaceutical application. However, several factors involved in the treatment such as the type and concentration of the chromosome doubling compound, as well as treatment times should be investigated further. This will improve the efficiency of tetraploid induction and minimize plant death. Moreover, biochemical purification and identification of the major bioactive compounds in crude extracts of line CC102 should be pursued further.

CHAPTER 3: Phytochemical alteration and new occurring compounds in hairy root cultures of *Mitracarpus hirtus* L. induced by phenylurea cytokinin (CPPU)

The transgenic hairy roots of *M. hirtus* were firstly successful established by *A. rhizogenes* A13. Moreover, these hairy roots have high potential to produce transgenic shoot with *rol* gene expression when applied light to the culture system. The results gain from this study performed that *M. hirtus* hairy root is an attractive system for secondary metabolite productions in the bio-reactor due to it has well proliferation, provides alternative source of pharmaceutical compound, and could be successfully induce new useful compounds after elicitation by CPPU.

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APPENDIX

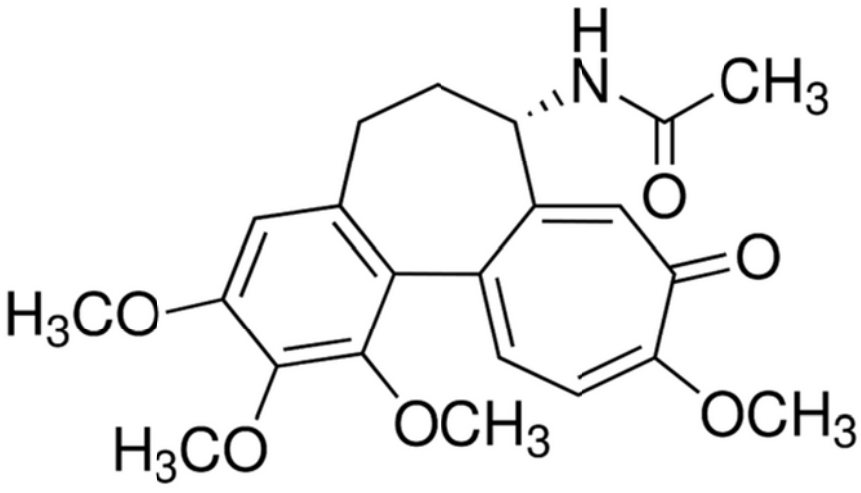

Figure 1 Colchicine (Sigma ®)

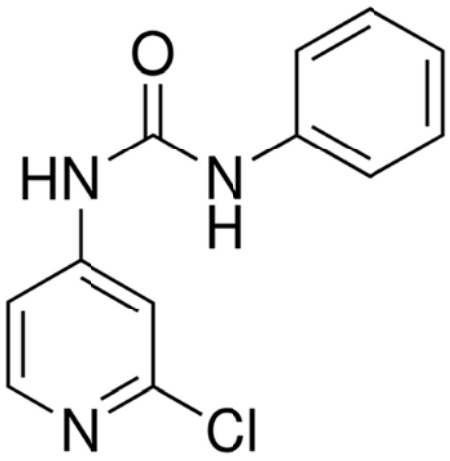

Figure 2 CPPU (2-chloro-4-pyridyl-N-phenylurea) (Sigma ®)

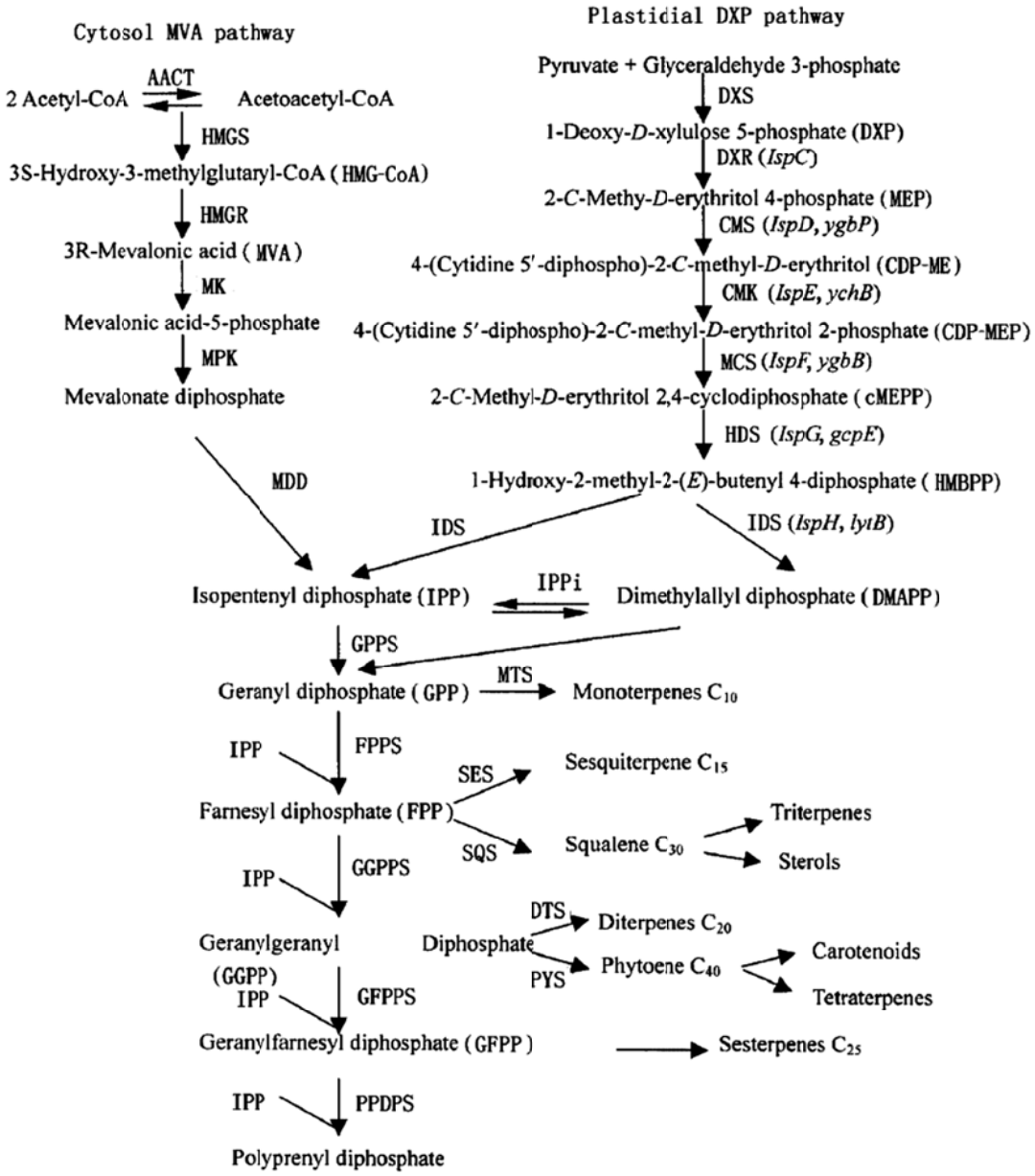

Figure 3 The isoprenoid biosynthetic pathway (Liu et al. 2005)

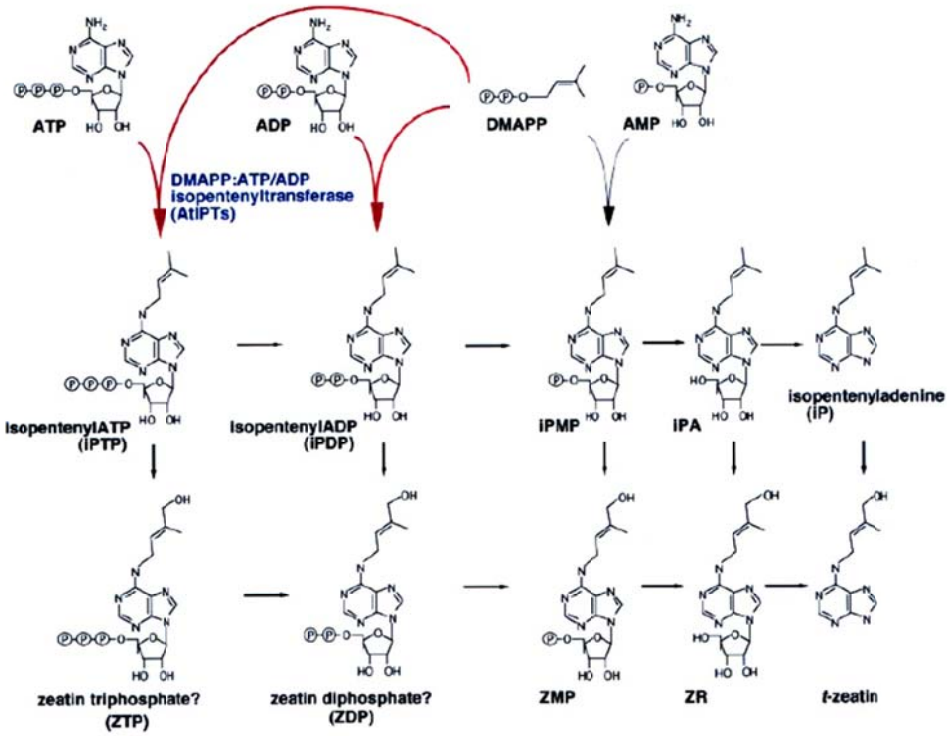

Figure 4 The cytokinin biosynthetic pathways in plants (Tatsuo 2011)

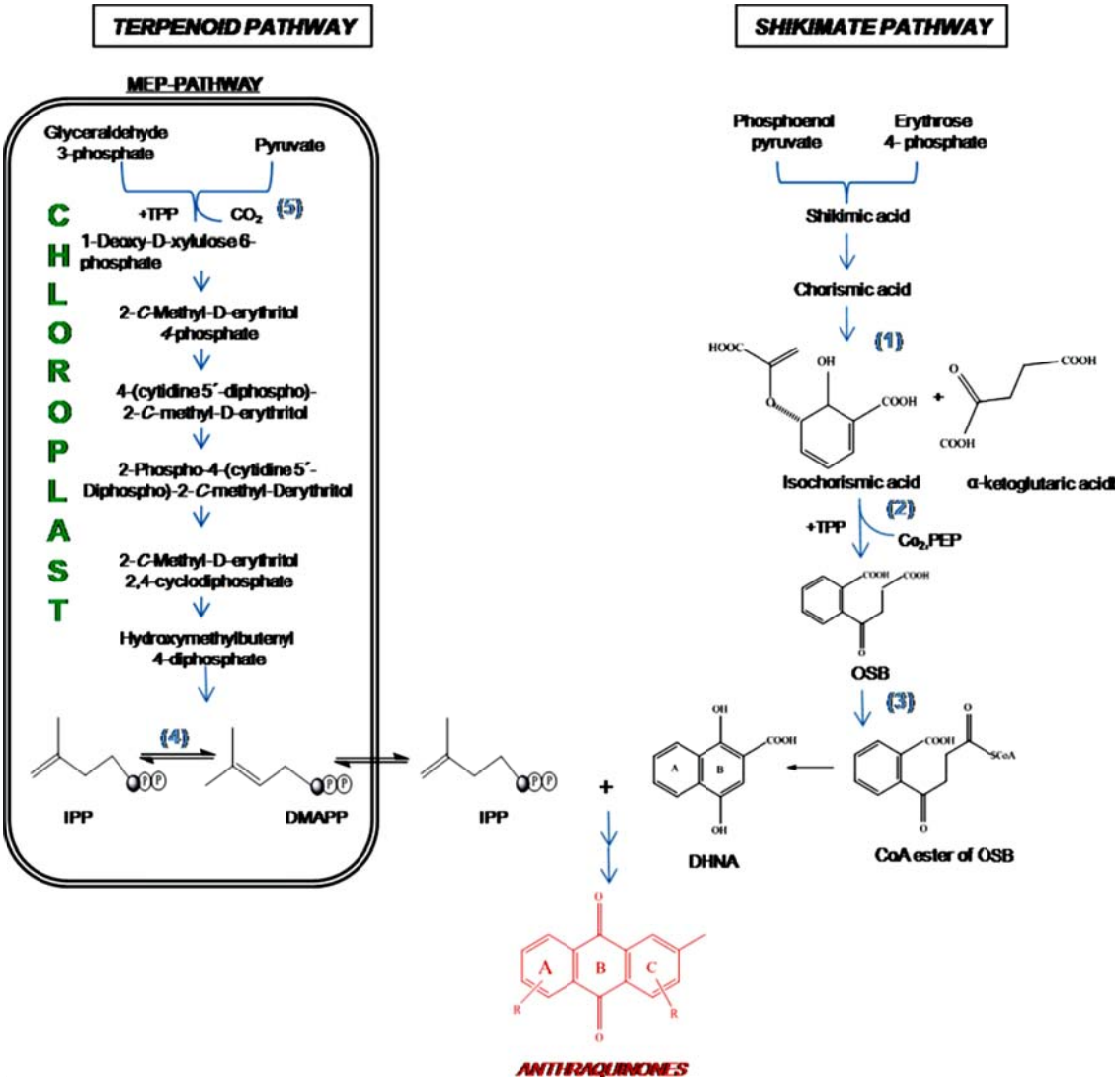

Figure 5 The biosynthetic pathways of anthraquinone (Quevedo et al. 2010)

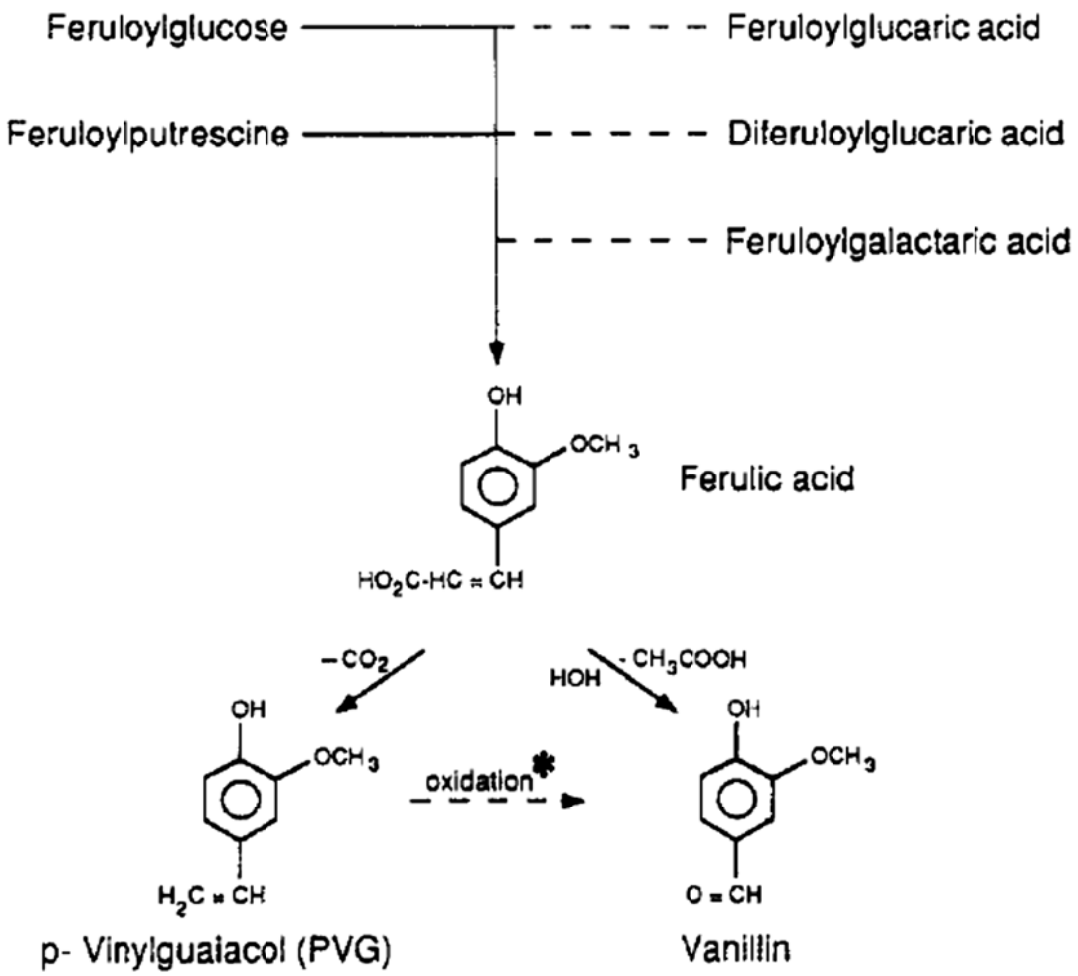

Figure 6 The biosynthetic pathways of *p*-vinylguaiacol (Peleg et al. 1992)

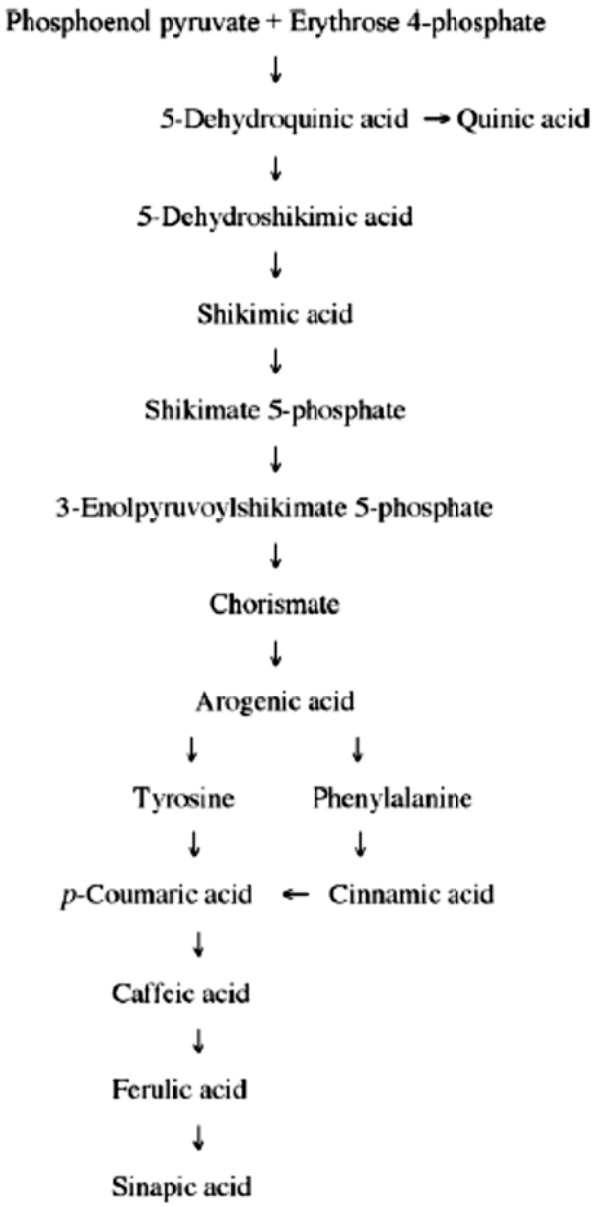

Figure 7 The biosynthesis of ferulic acid via shikimic acid pathway (Farah and Donangelo 2006)