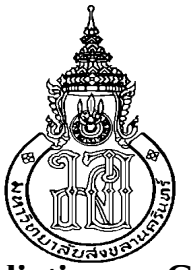

Effects of Gamma Ray Irradiation on Growth and Naphthoquinone Production of *Plumbago indica* Root Cultures

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Thesis Title Effects of Gamma Ray Irradiation on Growth and Naphthoquinone Production of *Plumbago indica* Root Cultures

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ABSTRACT

Plumbagin (5-hydroxy-2-methyl-1,4-napthoquinone) is a major active constituent of *Plumbago indica* L. (Plumbaginaceae) roots. It possesses various pharmacological activities that have been shown to assist the treatment of various diseases. Root cultures of *P. indica* were established from young leaf explants of *P. indica* on solid Gamborg's B5 medium supplemented with 0.1 mg/L α-naphthalene acetic acid (NAA). The root cultures were maintained in liquid B5 medium supplemented with the same hormonal composition. Time courses of growth and plumbagin production of the root cultures were determined. The root cultures reached the stationary phase at day 20, and gave the highest dry biomass (0.39 g/250-mL-flask), which was 3 times the biomass of the inoculated roots. Plumbagin was initially accumulated in a linear phase (after day 12) and actively biosynthesized until reaching the stationary phase. The highest content of plumbagin was observed at day 20 (0.86 mg/250-mL-flask).

The effects of low dose gamma ray irradiation (0, 5, 10, 15, 20, and 25 Gy) and cultures age for gamma ray irradiation (0, 5, 10, 15, and 20 days) on plumbagin production of *P. indica* root cultures was determined. Although all treated doses showed positive effects on plumbagin production, the dose of 20 Gy to the 12 days old root cultures appeared to show highest increase of plumbagin production (1.04 mg/g DW) when compared to all treated groups including control (0.89 mg/g DW)

The appropriate age of root cultures for maximum production of plumbagin (1.64 mg/g DW) was found to be 10 days, which was 2.94-fold higher than the level in the control (0.56 mg/g DW). However, treatment with 20 Gy to 5-day old root cultures resulted in a significant increase of dried root biomass, with a high plumbagin production (1.09 mg/g DW) that was 1.96-fold higher than the level in the control.

Based on the total biomass per cultured flask, the calculated amounts of plumbagin produced by the 5- and 10-day old treated roots were 0.59 and 0.37 mg/250-mL flask, respectively, which were 4.2- and 2.6 fold higher from the level in the control (0.14 mg/250-mL flask).

P. indica root sections were studied by SEM, focusing on the histological studies in the roots cultures exposed to gamma dose of 20 Gy. The *P. indica* histological anatomy consists of single layers of epidermis, cortex, endodermis and pericycle surrounding a vascular bundle. The 20 Gy treated root showed the radical expansion of root epidermal cells in the elongation zone of the roots and some burst in epidermal cells. Such effect of gamma ray irradiation causing radial expansion of epidermal cells initiating outgrowth of roots hairs or trichomes was observed.

The TLC and quantitative HPLC analyses of the untreated root cultures showed that plumbagin was the only major naphthoquinone produced by the treated and untreated *P. indica* root cultures. Different exposure with the gamma radiation exerted different effects on plumbagin production by the root cultures. But the treated and untreated root lacked the other two naphthoquinones. The treated root was subcultured further to observe the stability and the plumbagin production in the three generation batch. It was observed that in cycles of subculture the treated and untreated root showed healthy growth and stable plumbagin production.

This study concludes that the elicitation technique is a useful tool to stimulate production of plumbagin in *P. indica* root cultures. The exposure doses of elicitor and age of the root cultures play an important role in stimulation of plumbagin production. This study demonstrated that the treatment of *P. indica* root cultures with gamma ray irradiation can influence the plumbagin production. The treatment of *P. indica* root cultures on day 5- and 10-days was found to be suitable with further use of two phase culture. A high degree of stability for plumbagin production by the elicitated root cultures was achieved during three cycles of subculture.

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I would be very happy if my thesis could be of use for further studies. And I look forward eagerly to constructive feedback from the readers.

Amit Jaisi

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LIST OF ABBREVIATIONS

%	Percent
% RSD	Percentage relative standard deviation
˚C	Degree Celsius
/	Per
µg	Micro gram
µL	Micro liter
µm	Micrometer
µM	Micro molar
cm	Centimeter
e.g.	Exempli gratia, for example
et al.	et alia, and others
etc.	et cetera, and other things
g	Gram
GHz	Gigahertz
Gy	Gray
h	Hour
IC_{50}	50% Inhibitory concentration
kg	Kilogram
kPa	Kilopascal
L	Liter
LC_{50}	50% Lethal dose
mg	Milligram
MHz	Megahertz
min	Minute
mL	Milliliter(s)
mm	Millimeter(s)
mM	Millimolar
MICs	Minimum inhibitory concentrations
MBCs	Minimum bactericidal concentrations

LIST OF ABBREVIATIONS (CONTINUED)

pH	Negative logarithm of the hydrogen ion concentration
ppm	Part per million
s	Seconds
SE	Standard error of the mean
v/v	Volume by volume
W	Watt
w/w	Weight by weight
w/w	Weight by volume
2,4-D	2,4-Dichlorophenoxyacetic acid
B5	Gamborg's B5 medium
BA	6-Benzyladenine
NAA	α-Naphthalene acetic acid
HPLC	High performance liquid chromatography
DW	Dry weight
FW	Fresh weight
M	Molar
mg/g DW	Milligram per gram dry weight
PDA	Photodiode array
TLC	Thin layer chromatography
UV	Ultra violet

CHAPTER 1

INTRODUCTION

1. Background and Rationale

Owing to the concerns for health and well-being of humanity, plants have been selected as the ultimate sources for drugs, colors, spices and fragrances. The features of plants are the focal point of curiosity since the dawn of early civilization. A typical feature of plants is the production and accumulation of secondary metabolites. Secondary metabolites are compounds biosynthetically derived from primary metabolites. They are not essential for energy metabolism and life. These products appear to be important in the interactions between the plant and its environment (Wink, 1988; Harborne, 1993; Wink, 2006). Therefore, secondary metabolites are of major interest because of their different functions and their impressive biological activities ranging from antimicrobial, antibiotic, insecticidal and hormonal properties to highly important pharmacological activities (Stöckigt *et al.*, 1995).

Nowadays, many of the plants containing high-value compounds are difficult to cultivate and are becoming endangered because of over-harvesting (Rates, 2001). Plant cell cultures and biotechnological approaches are attractive alternative sources to whole plants for the production of high-value secondary metabolites and in improving the productivity. When compared to traditional agricultural growth, plant tissue cultures of medicinal plants offer a number of year-round, continuous productions of plant medicinal compounds under highly controlled conditions. As the *in vivo* production of secondary metabolites by plants are highly influenced by plant growth environment factors such as climactic and soil conditions, pathogen attack and herbivores (Wink, 2003).

Plant cell and tissue cultures are potentially used as an alternative source to the whole plants for production of valuable phytochemicals. The biosynthetic capacities of plants are exploited *in vitro* using plant cells and tissue systems analogous to microbial cells in fermentation process. However, the important

requirement for the improvement of secondary metabolite synthesis in plants is the understanding of the metabolic pathways and the enzymology of the biosynthesis of particular products (Heike and Dietrich, 1995). In general, several strategies have been used to increase yield of secondary metabolites including: a) selection of the high-yield lines; b) change in formulation of medium composition i.e. carbon source, nitrogen, phosphate, plant growth regulators; c) varying in culture conditions i.e. pH, temperature, light; d) cultivation strategies i.e. immobilization, organ culture, hairy roots and e) applying specialized techniques such as elicitation, genetic transformation and metabolic and integrated bioreactor engineering (Dörnenburg and Knorr, 1995; Ramachandra and Ravishankar, 2002). Many studies have described successful strategies for the increase in production of secondary metabolites by elicitation techniques (Namdeo, 2007).

Secondary metabolites are stimulated by several types of factors. These molecules are termed as ‘elicitors’ and the phenomenon is known as ‘elicitation’. Elicitors produced within plant cells are termed as endogenous elicitors, while those produced by microorganisms are called exogenous elicitors. Elicitations have been found to be one of the most effective biotechnological approaches to induce or enhance biosynthesis of metabolites (Radman *et al*., 2003). Elicitors may be biotic or abiotic compounds which are able to trigger plant defense mechanisms such as hypersensitive response, production of reactive oxygen species and activation of defense-related genes as well as phytoalexin synthesis (Smith, 1996; Ebel *et al*., 1998). Examples of biotic elicitors are bacterial and fungal cell wall; abiotic elicitors are UV light, temperature and heavy metals (Singh, 1999; Chong *et al*., 2005). Various studies and a number of reports show the use of UV radiation, gamma radiation, low-energy ultrasound, hormone, and precursor feeding to regulate production of secondary (Möhle *et al.*, 1985; Lin and Wu, 2002; Edahiro *et al.*, 2005; Chung *et al.*, 2006; Koobkokkruad *et al.*, 2008; Khattak and Simpson, 2010). Several types of products related to naphthoquinones have been successfully elevated by elicitation (Fu and Lu, 1999; Komaraiah *et al.*, 2003; Sakunphueak and Panichayupakaranant, 2010).

In the last decade, gamma ray irradiation had drawn attention as a new and rapid method to improve the qualitative and quantitative nature of many crops. It has

been reported that relatively low-dose ionizing irradiation on plants and photosynthetic microorganisms are manifested as accelerated cell proliferation, germination rate, cell growth, enzyme activity, stress resistance and crop yields (Chakravarty *et al*., 2001). Gamma rays influence plant growth and development by inducing cytological, biochemical, physiological, genetic and morphogenetic changes in cells and tissue (Gunckel *et al*., 1961). It has been reported that the irradiation of plant tissues with gamma rays leads to the intracellular generation of active oxygen radicals and hydrogen peroxide, which are known to play an important role in signaling plant defense responses (Apostol *et al.*, 1989).

Plumbago indica L. is a medicinal plant belonging to the family Plumbaginaceae. Medicine preparations from the roots of *P. indica* are claimed to have thermogenic, anthelmintic, anti-inflammatory, abortifacient, anti-periodic, carminative, digestive, nerve stimulatory and rejuvenating properties (Joy *et al.*, 1998). The roots of this plant have commercial importance as a major source of plumbagin. Plumbagin is well known for its broad ranges of pharmacological activities such as antibacterial (Kaewbumrung and Panichayupakaranant, 2011), anticancer (Wang *et al.*, 2008), antimicrobial (Park *et al.*, 2006), antiprotozoan (Fournet *et al.*, 1992), anthelmintic (Atjanasuppat *et al.*, 2009), and antifertility (Kini *et al.*, 1997) activities. *P. indica* roots are being used increasingly as ingredients in marketed phytomedicines. However, this plant grows quite slowly and it takes at least two years for the roots to be suitable for use (Kitanow and Pashankov, 1994). Thus, many studies have focused on the production of plumbagin using *in vitro* culture techniques that offer an alternative for the production of such pharmaceutically important compound. An establishment of *P. indica* root cultures, using a selection of plumbagin high yielding plant as explants for initiation has been previously described (Panichayupakaranant and Tewtrakul, 2002). But, the root cultures still produced low plumbagin level. Production of plumbagin through suspension culture, immobilization and elicitation has also been reported in *P. indica* (Komaraiah *et al.*, 2001; Komaraiah *et al.*, 2002; Komaraiah *et al.*, 2003; Komaraiah *et al.*, 2004). However, they still had several problems in the production of plumbagin by the cell cultures such as the instability and slow growth of the cell lines and low yields of plumbagin.

According to the literature review; it indicates a promising advantage in using elicitation techniques, especially the gamma ray irradiation, for increased secondary metabolite production by plant tissue cultures. However, there is very limited information on the enhancement of plumbagin production induced by gamma ray irradiation. The main aims of this study are therefore to use gamma ray irradiation as the elicitor for increased plumbagin production in *P. indica* root cultures as well as optimization of gamma ray dose and age of root cultures for elicitor treatment in order to obtain appropriate elicitation conditions that produce a higher content of plumbagin. Moreover, the effect of gamma rays on the chemical profile of naphthoquinone production of the root cultures was also determined.

2. Objectives

The main objectives of this study were;

1. To establish the root cultures of *P. indica* and to determine of their time courses of growth and naphthoquinone production
2. To investigate the effects of gamma ray irradiation on growth and naphthoquinone production of *P. indica* root cultures
3. To optimize gamma ray irradiation conditions for the increase in plumbagin production
4. To determine naphthoquinone production profile of the root cultures
5. To investigate stability of the elicitated root cultures on growth and plumbagin production

CHAPTER 2

LITERATURE REVIEW

1. *Plumbago*

Medicinal plants are gaining great interest and value in pharmaceutical industries for the production of high valued secondary compounds (Rout *et al.*, 2000). *Plumbago indica* L. is a shrub belonging to the family Plumbaginaceae whose roots are the main source of plumbagin, a naphthoquinone derivative of commercial interest for its wide range of pharmacological properties.

Plumbago is a genus in Plumbaginaceae family. It's characterized by herbs perennial or rarely annual, rarely shrubs. Stems are usually branched and growing to 0.5-2 m tall. The leaves are spirally arranged, simple, entire, 0.5-12 cm long, with a tapered base and often with a hairy margin. The flowers are white, blue, purple, red, or pink, with a tubular corolla with five petal-like lobes; they are produced in racemes. The flower calyx has glandular hairs, which secrete sticky mucilage that is capable of trapping and killing insects. The ovary is ellipsoid, ovoid, or pyriform. There are about 25 species around the world, native to warm temperate to tropical regions, but 2 species including *Plumbago indica* L. and *Plumbago zeylanica* L. are found in Thailand (Schlauer, 1997; Schmelzer and Gurib-Fakim, 2008).

2. *Plumbago indica* L.

Scientific name: *Plumbago indica* L. (Figure 1)

Family name: Plumbaginaceae

Synonym: *Plumbago rosea* L.

English name: Rosy-flowered Leadwort

Thai name: Chettamuun Phloeng Daeng (เจตมูลเพลิงแดง)

Figure 2.1 *Plumbago indica* L.

3. Botanical aspects of *P. indica* L.

P. indica L. (Figure 1) or Chettamuun Phloeng Daeng (Thai name), a native of South Asia, is a small shrub. *P. indica* is a shrubby and evergreen plant, which frequently grows to the height of 0.5 – 2 m. Petiole base without auricles; leaf blade narrowly ovate to elliptic-ovate, papery, base rounded to obtuse, apex acute. Inflorescences 35–90 flowered; peduncle 1–3 cm, not glandular; rachis 10–40 cm, not glandular; bracts ovate, 2–3 × 1.5–2 mm, apex acuminate; bractlets obovate-elliptic to ovate, 2–2.5 × 1.5–2 mm, apex acute. Flowers are heterostylous. Calyx 7.5–9.5 mm, glandular almost throughout, tube is 2 mm in diameter at middle. Corolla red to dark red, tube is 2–2.5 cm, apex rounded and mucronate. Anthers blue, 1.5–2 mm. Ovary ellipsoid-ovoid, indistinctly angular. Style basally pilose; short-styled forms with style arms partly exserted, stigmatic glands without enlarged apex; long-styled form with

style arms completely exserted from corolla throat, stigmatic glands capitates (Schmelzer and Gurib-Fakim, 2008; Kaewbumrung, 2010).

4. Ecology and propagation of *P. indica*

P. indica L. can be propagated by following either sexual or asexual reproduction. However, as it produces very small amount of seed so, seed germination is poor. The asexual propagation, both root and stem cutting are the most commonly used propagation.

Plumbagin (5-hydroxy-2-methyl-1, 4-naphthoquinone) is a naturally occurring yellow pigment found in the plants of the Plumbaginceae, Droseraceae, Ancistrocladaceae and Dioncophyllaceae families. Plumbaginceae is found in Africa, many parts of Asia and Europe while Droseraceae (sundew) family is found in many temperate and tropical regions of the world (Chuntaratin, 2006).

5. Medicinal properties of *P. indica* L

In Thai traditional medicine, *P. indica* roots were used for gastric stimulant, flatulence, hemorrhoid, appetizer and adaptation of uterus after delivery. In large doses, it is acro-narcotic poison. Locally, it is used for wound healing, tinea versicolor and ringworm.

Roots of *P. indica* L. have been used as raw material for extracting plumbagin in China and other countries. The root is acrid, vesicant, abortifacient and stimulant. Applied in blended oil it is used externally or internally in rheumatism and paralytic afflictions. The root is also a powerful sialogogue and a remedy for secondary syphilis, leprosy and leucoderma. The milky juice of the plant is also used against ophthalmia and scabies (Joy *et al.*, 1998).

In eastern Africa and India *P. indica* was traditionally used for gastric stimulant, abortifacient and oral contraceptive. An infusion of roots is taken to treat dyspepsia, colic, cough and bronchitis. A liniment made from bruised root mixed with a little vegetable oil is used as a rubefacient to treat rheumatism and headache (Schmelzer and Gurib-Fakim, 2008).

6. Chemical constituents of *P. indica*

As reported by Farnsworth (1999), several groups of phytochemical have been found in different plant parts of *P. indica* L. The quinone comprising plumbagin was identified in roots, root bark and aerial parts of this plant. A pharmacologically active compound derived from *P. indica* includes naphthoquinones, coumarins, flavonoids, tannins, alkaloids, saponins and phenolic compounds (Ariyanathan *et al.*, 2010; Lenora *et al.*, 2012). *P. indica* L. roots were studied and reported that it consist of high amount of plumbagin and other two naphthoquinones including 3, 3'-biplumbagin and elliptinone (Kaewbumrung and Panichayupakaranant, 2011) (Figure 2.2). Other chemical constituents found in this species are shown in Table 2.1.

Table 2.1 Chemical constituents of *Plumbago indica*

	Chemicals	Plant parts	References
1	**Naphthoquinones**		
	Plumbagin	Roots, Aerial and barks	(Dinda *et al.*, 1995; Kaewbumrung and Panichayupakaranant, 2011)
	Droserone	Roots	(Dinda *et al.*, 1995)
	Elliptinone	Roots	(Kaewbumrung and Panichayupakaranant, 2011)
	Zucylanone	Roots	(Dinda *et al.*, 1995)
	3,3' -biplumbagin	Roots	(Kaewbumrung and Panichayupakaranant, 2011)
	Isoshinanolone		(Dinda *et al.*, 1995)
	Roseanone	Roots	(Dinda *et al.*, 1995)
2	**Flavonoids**		
	6-hydroxyplumbagin	Aerial	(Dinda and Chel, 1992)
	Plumbaginol	Aerial	(Okeyo, 2006)

Table 2.1 Chemical constituents of *P. indica* (continued)

	Chemicals	**Plant parts**	**References**
	Myricetin-3,3',5',7-tetra methyl ether	Roots	(Ariyanathan *et al.*, 2010)
	Ampelopsin-3',4',5'7-tetramethyl ether	Roots	(Ariyanathan *et al.*, 2010)
3	**Steroids**		
	β-sitosterol	Aerial	(Dinda *et al.*, 1999
	Stigmasterol	Aerial	(Dinda *et al.*, 1999)
	Campesterol	Aerial	(Dinda *et al.*, 1999)
4	**Leucodelphinidin**	Aerial	(Okeyo, 2006)
5	**Plumbagic acid lactone**	Roots	(Dinda *et al.*, 1999)
6	**Physcion-β-D-glucopyranoside**	Roots	(Dinda *et al.*, 1999)
7	**Sitosterol glycoside**	Roots, Bark	(Dinda *et al.*, 1999)
8	**Tannin**	Roots, Bark	(Dinda *et al.*, 1999)
9	**Azalein**	Flowers	(Harborne, 1962)
10	**Carboxylic Acids**		
	Plumbagic acid	Roots	(Ariyanathan *et al.*, 2010)
	Roseanoic acid	Roots	(Ariyanathan *et al.*, 2010)
11	**Flavonyl methyl ethers**		
	Azaleatin		(Ariyanathan *et al.*, 2010)
	Cyanin		(Ariyanathan *et al.*, 2010)
12	**Fatty Acids**		
	Palmitic acid		(Ariyanathan *et al.*, 2010)
	Myricyl palmitate		(Ariyanathan *et al.*, 2010)

Table 2.1 Chemical constituents of *P. indica* (continued)

13	**Triterpene**		
	α-amyrin	Roots	(Ariyanathan *et al.*, 2010)
	β-amyrin	Roots	(Ariyanathan *et al.*, 2010)

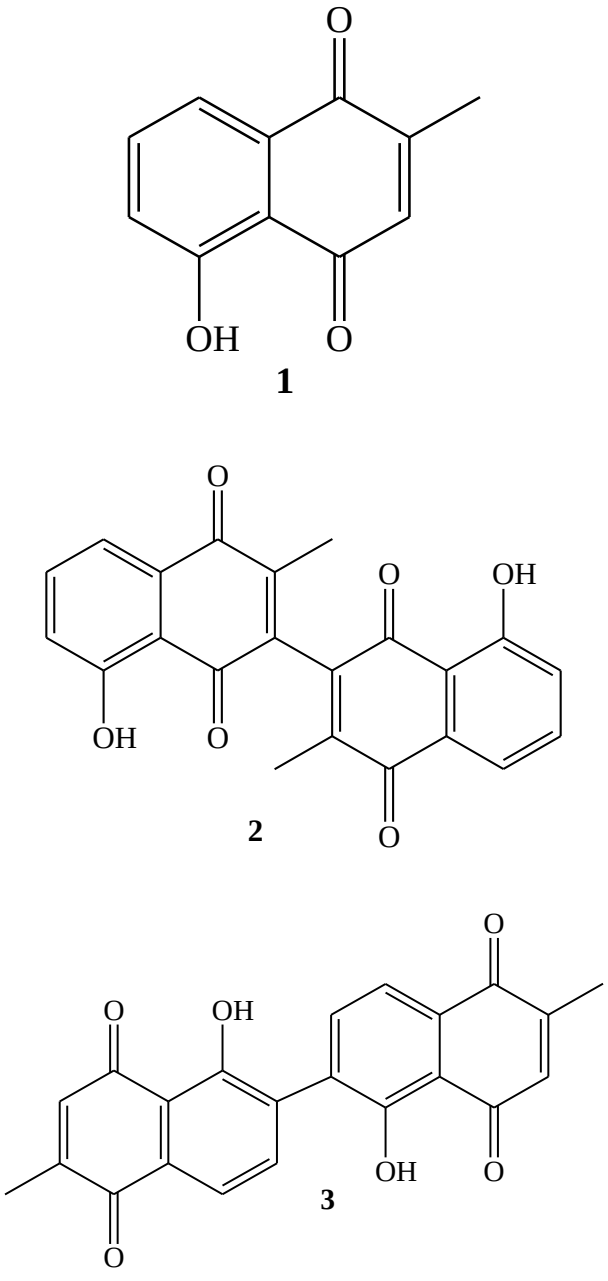


Figure 2.2 Chemical structures of naphthoquinone from *P. indica* roots plumbagin (**1**), 3, 3'-biplumbagin (**2**) and elliptinone (**3**)

7. Pharmacological properties of *Plumbago indica*

7.1 Antifungal activity

Hydroalcoholic (80% ethanol) extract of *P. indica* roots possess potent antifungal activity against *Aspergillus niger* and *Candida albicans* (Valsaraj *et al.*, 1997). In addition, plumbgin had been reported as the active compound against *C. albicans* with MIC and MFC (Minimum Fungicidal concentration) values of 0.78 and 1.56 µg/ml (Paiva, S. R. d. *et al.*, 2003).

7.2 Antibacterial activity

The Hydroalcoholic (80% ethanol) extract of *P. indica* roots was found to exhibit antibacterial activity against *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *S. aureus* with MIC value of 6.25 mg/ml for B. subtilis and 12.5 mg/ml for *P. aeruginosa*, *E. coli* and *S. aureus* (Valsaraj *et al.*, 1997). Moreover, plumbagin had been reported as the active compound against *S. aureus* with MIC and MBC values of 1.56 and 25 µg/ml respectively (Paiva, S. R. d. *et al.*, 2003).

7.3 Antiparasite activity

P. indica roots extract showed a macrofilaricidal property against *Setaria digitat*a, a filarial parasite of cattle. Complete inhibition of motility was observed at concentration range between 0.02 and 0.05 mg/ml. Fractionation of the crude extract resulted in the isolation of the active molecule plumbagin (Paiva, S. R. *et al.*, 2003).

7.4 Antifertility activity

Acetone extract of *P. indica* stems exhibited the activity in interrupting the normal estrous cycle of female Albino rats at two dose levels, 200 and 400 mg/kg. The rats exhibited prolonged diestrous stage of the estrous cycle with consequent temporary inhibition of ovulation. The anti-ovulatory activity was reversible on withdrawal of the extract. The effective acetone extract was further studied on estrogenic functionality in rats. The acetone extract showed significant

estrogenic and antiestrogenic activity. Histological studies of the uteri further confirmed the estrogenic activity of the acetone extract (Sheeja *et al.*, 2008).

8. Plumbagin chemical structure and properties

Plumbagin, 2-methoxy-5-hydroxy-1, 4-naphthoquinone ($C_{11}H_8O_3$), is a natural product found in roots of *P. indica* with a molecular weight of 188.18. Plumbagin occurs as yellow pigment and its melting point is 78-79 ºC. It is slightly soluble in hot water and well soluble in alcohol, acetone, chloroform, benzene and acetic acid. It is highly toxic and corrosive. The structure of plumbagin is shown in Figure 2.2.

9. Pharmacological properties of plumbagin

Plumbagin, the most active naphthoquinone derived from the species of *Plumbago*, *Drosera* and *Diospyros*, has been widely studied for its pharmacological activities. It has been reported that, in low dose it is a soporific and stimulates the central nervous system, while in high doses may even cause death from respiratory failure and paralysis. Plumbagin and its related naphthoquinones available naturally are commercially important for its pharmacological activities such as, anti-tumor (Lin *et al.*, 2003), anticancer (Parimala and Sachdanandam, 1993), anti-intestinal carcinogenesis (Sugie *et al.*, 1998), anti-microbial (Wang and Huang, 2005, Durga *et al.*, 1990; Didry *et al.*, 1994), antimalarial (Likhitwitayawuid *et al.*, 1998), antifertility (Bhargava, 1984) and cardiotonic activities (Itoigawa *et al.*, 1991). Furthermore, it has been reported that the plumbagin showed interesting pharmacological activities that includes the following:

9.1 Antitumor activity

Plumbagin exhibited anticancer activity against melanoma cell lines (Bowes cell) and breast cancer cells line (MCF-7) with IC_{50} values of 1.39 and 1.28 µM, respectively (Nguyen *et al.*, 2004). For breast cancer cells, plumbagin inhibited cell proliferation by inducing cells to undergo G2-M arrest and autophagic cell death.

Blockade of the cell cycle was associated with increase p21/WAF1 expression and Chk2 activation, and reduced amounts of cyclin B1, cyclin A, Cdc2 and Cdc25C. Plumbagin also reduced Cdc2 function by increasing the association of p21/WAf1/Cdc2 complex and the levels of inactivated phosphor-Cdc2 and phosphor-Cdc25C by Chk2 activation (Kuo *et al.*, 2006).

Anticancer effect of Plumbagin had been reported against human non-small cell lung cancer cells A549 with IC_{50} value of 11.69 uM. It exhibited effective cell growth inhibition by inducing cancer cells to undergo G2-M phase arrest and apoptosis. Blockade of cell cycle was associated with increased levels of p21 and reduced amounts of cyclin B1, Cdc2 and Cdc25C. Plumbagin treatment also enhanced the levels of inactivated phosphorylated Cdc2 and Cdc25C. Bloackade of p53 activity by dominant-negative p53 transfection partially decreased plumbagin-induced apoptosis and G2-M arrest, suggesting it might be operated by p53-dependent and independent pathway. Plumbagin treatment triggered the mitochondrial apoptotic pathway indicated by a change in Bax/Bcl-2 ratios, resulting in mitochondrial membrane potential loss, cytochrome-*c* release, and caspase-9 activation (Hsu *et al.*, 2006).

Furthermore, Kai-Hong Xu and his group examined whether the effect of plumbagin was valid *in vivo*. NB4 cells were injected into the flanks of NOD/SCID mice, because the anti-tumor effect could be quantitatively and pathologically assessed. They found out that plumbagin can inhibit the proliferation of NB4 cells with an IC_{50} value of ~9 μM; suggesting APL (Acute promyelocytic leukemia) cells are more susceptible to plumbagin than lung cancer and melanoma cells. The results showed that intraperitoneal injection of plumbagin (2 mg/kg body weight/daily) for 3 weeks resulted to a 64.49% reduction of tumor volume compared with the control (Xu and Lu, 2010).

9.2 Anti-inflammatory activity

Plumbagin exhibited an immunomodulatory effects by inhibition of T cell proliferation in response to polyclonal mitogen Concanavalin A (Con A) by blocking cell cycle progression (IC_{50} value of 50 μM). It also suppressed expression of early and late activation markers CD69 and CD25, respectively in activated T cells.

The inhibition of T cell proliferation by plumbagin was accompanied by a decrease in the levels of Con A induced IL-2, IL-4, IL-6 and IFN-γ cytokines (Checker *et al.*, 2009).

In addition, plumbagin was found to be a therapeutic potential for its anti-allergic and anti inflammatory effects for the first time in human PMBC (Peripheral blood mononuclear cells). The allergen-specific immune responses like lymphocyte proliferation and cytokine secretion were studied in vitro using PMBC isolated from both allergic and non allergic individuals. Although, some allergens induced significant lymphocyte proliferation in vitro, allergen-induced cytokine secretion except that of TNF-α was not seen. Significant higher ratio of secreted IL-4/IFN-γ cytokines was observed in PMBC isolated from allergic subjects in response to PHA. Plumbagin (vitamin K3 analogue) completely inhibited PHA-induced cytokine production in PBMC, in both allergic and non-allergic individuals. Plumbagin modulated the levels of intracellular reactive oxygen species and glutathione and suppressed PHA induced activation of NF-κB in human PBMC (Kohli *et al.*, 2011).

9.3 Antimalarial activity

It has been reported that plumbagin exhibited anti-*Plasmodium falciparum* activity by inhibition of isolated *P. falciparum* enzyme, succinate dehydrogenase (SDH), with IC_{50} value of 5 mM. It also inhibits in vitro growth of *P. falciparum* with IC_{50} value of 0.27 mM (Paiva, S. R. *et al.*, 2003).

In addition, Sreelatha and her coworker (2010) conducted test of all the napthaquinone derivatives from *Plumbago capensis* for their mosquito larvicidal activity against fourth instar larvae of *Aedes aegypti* and compared with that of rotenone. Among the tested compounds, isoshinanolone and plumbagin showed excellent toxicity with IC_{50} values of 1.26 and 5.43 µg/mL (Sreelatha *et al.*, 2010).

9.4 Antibacterial activity

Plumbagin has been reported as an Anti-*Helicobacter pylori* agent with MIC value of 4.0 µg/mL, which was more potent than that of metronidazole (MIC value of 32 µg/mL) (Park *et al.*, 2006). Farr and his coworker (1985) reported

on an antibacterial activity of plumbagin against wild type *E. coli* strain AB1157 with 99.9% killed by exposure to 1.0 mM plumbagin for 1 hour at 37ºC. Antibacterial mechanism of plumabgin may be due to its toxicity by generated active oxygen species and may damage DNA besides a pathway *via* H_2O_2.

In contrast, Jamieson and his co-workers (1994) conducted tests in wild-type strain *Saccharomyces cerevisiae* S150-2B and mutated strains using disruption mutations in the genus encoding of two superoxide dismutases, Cu/ZnSOD *(SOD1)* and mitochondrial MnSOD *(SOD2).* The result showed that the *SOD2* strain was 100-fold more sensitive to plumbagin than its parent, while the sensitivity of the *SOD2* strain to plumbagin was indistinguishable from that of the wild type strain. Thus Cu/ZnSOD was the principle superoxide dismutating genes target.

Kamal and his coworker (1995) conducted *in vivo* anti- *S. aureus* test in female mice and showed that plumbagin increase its activity up to 8 weeks with 25 µg/Kg body weight, due to its ability to stimulate the response on oxygen radical release by macrophages. While at high dose (50 µg/Kg body weight), it has direct inhibitory activity against *S. aureus*.

Furthermore, Kuete and his co-worker (2011) conducted antimicrobial activities of various natural products including plumbagin against a collection of Gram-negative multidrug-resistance (MDR) bacteria. The activities of plumbagin increased significantly and exhibited better activity of all natural compounds tested against all studied MDR bacteria. MIC values < 10mg/L was obtained for plumbagin against E. coli AG100A and $AG100A_{Tet.}$ (Kuete *et al.*, 2011)

9.5 Mutagenic activity

Plumbagin was reported having an antimutagenic activity against *Salmonella typimurium* TA98 when induced by 2-nitrofluorene (2NF), 3-nitrofluoranthene (3-NFA) and 1-nitropyrene (1-NP) (Edenharder and Tang, 1997). Moreover, for *Escherichia coli* WP2s *(uvrA trpE)*, plumbagin was not mutagenic when presence of plasmid pKM101 (Kato *et al.*, 1995).

9.6 Antifertility activity

Plumbagin containing albumin microspheres were implanted to 20 days pregnant albino rats and found that their ovaries showed clear inhibition of growth of graffian follicles and degeneration of the mature follicles, and corpus luteum were observed and resulted in the failure to conceive, the antifertility action of plumbagin seemed to be related to its antiovulatory action (Kini *et al.*, 1997).

9.7 Cardiotonic action

Plumbagin produced a triphasic inotropic response in guinea-pig papillary muscle. Plumbagin did not cause any positive inotropy under anoxic conditions, and the positive inotropic effects were markedly inhibited by oxidative phosphorylation uncouplers (Itoigawa *et al.*, 1991).

9.8 Immunosuppressive

Plant containing plumbagin (PLB) was reported to have used as a treatment of chronic immunologically-based diseases. However, McKallip and his co-worker conducted a test and found out that plumbagin has significant immunosuppressive properties which are mediated by generation of ROS, upregulation of Fas, and the induction of apoptosis. Splenocytes from C57BL/6 mice cultured in the presence of 0.5 μM or greater concentrations of PLB significantly reduced proliferative responses to mitogens, including anti-CD3 mAbs, concanavalin A (Con A), lipopolysaccharide (LPS) and staphylococcal enterotoxin B (SEB) in vitro. Exposure of naïve and activated splenocytes to PLB led to a significant increase in the levels of apoptosis. In addition, PLB treatment led to a significant increase in the levels of reactive oxygen species (ROS) in naïve and activiated splenocytes.

Furthermore, treatment with the ROS scavenger, N-acetylcysteine (NAC), prevented PLB-induced apoptosis, suggesting a role of ROS in PLB-induced apoptosis'. PLB-induced apoptosis led to ROS-mediated activation of both the extrinsic and intrinsic apoptotic pathways. In addition, plumbagin led to increase expression of Fas. Finally, treatment of mice with PLB (5 mg/Kg) led to thymic and splenic atrophy as well as a significant suppression of the response to SEB and dinitroflourobenzene (DNFB) in vivo (Roberts and Paul, 2006).

10. Extraction and quantification of plumbagin

The extraction and determination of plumbagin from *Plumbago spp*. have been reported by many researchers. Kitanov and Pashakov (1994) isolated plumbagin using HPLC technique from petroleum ether extract of *P. europaea*, while Gupta et al. (1993) purified from the root of *P. zealanica* L. using preparative silica gel column chromatography. Plumbagin from *P. zealanica* L. and *P. indica* L. roots has also been extracted with methanol and determined by HPLC or TLC densitometric method (Choosakul, 2000; Komaraiah et al., 2001; Panichayupakaranant and Tewtrakul, 2002). The extraction and quantification of the plumbagin in this research work is followed based on the method established and validated by Kaewbumrung and Panichayupakaranant (2011).

11. Biosynthetic pathway of Plumbagin

Plant secondary metabolites are formed from glucose metabolism intermediated by the Shikimic, Acetate and Amino acid pathways (Geissman and Crout, 1967). Naphthoquinones mostly are derived from Shikimate pathways. Plumbagin biosynthesis was studied quit long time ago and was found it originates from acetate (6 C_2) units and is derived via polyketide pathways (Durand and Zenk, 1971; Durand and Zenk, 1974). But the proper biosynthetic metabolism of the plumbagin synthesis is yet to be known. It was postulated that plumbagin and biosynthetically related naphthoquinones and tetralones isoshinanolone are synthesized by polyketide synthase (PKSs) via the acetate polymalonate pathway (Bringmann *et al.*, 1998, Bringmann and Feineis, 2001). This suggested that the biosynthesis is carried out by a polyketide synthase using acetyl-CoA as starter and followed by performing five condensation reactions with malonyl-CoA.

However, recent investigations with PKS cloned from *P. indica* (Springob *et al.*, 2007) and *Drosophyllum lusitanicum* (Jindaprasert *et al.*, 2008) were studied and postulated mechanism of plumbagin biosynthesis comparison with the reaction of chalcone synthase (CHS). The PKS involved in plumbagin biosynthesis presumably catalyzes the decarboxylative condensation of acetyl-CoA with five molecules of

malonyl-CoA. The oxygen of the third acetate unit is probably removed by a polyketide reductase (PKR) prior to the first cyclization, and one carbon is lost by decarboxylation. The available studies and data suggest that there might be actually a PKS in plumbagin biosynthesis, but this needs to be proven by other means, e.g. by transgenic expression or deleting the gene from the plant.

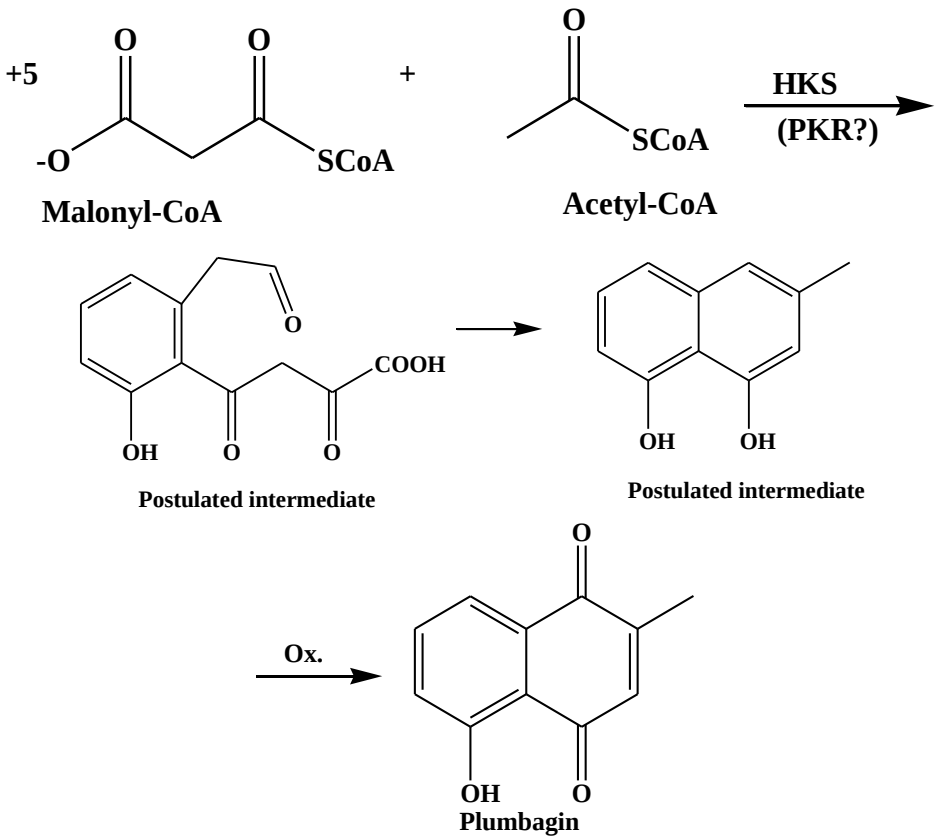


Figure 2.3 Biosynthetic pathway of plumbagin (Jindaprasert *et al.*, 2008)

12. Production of secondary metabolites using plant tissue culture

Studies on plant secondary metabolites have been increasing over the last 50 years. These molecules are known to play a major role in the adaptation of plants to their environment, and also represent an important source of active pharmaceuticals. Plant cell culture technologies were introduced at the end of the 1960s as a tool for both studying and producing plant secondary metabolites. Many studies have been under taken with the objective of improving the *in vitro* production of plant secondary compounds. Undifferentiated cell cultures such as callus and cell

suspension have been mainly studied, but a large interest has also been shown in hairy roots and other organ cultures (Bourgaud *et al.*, 2001). Among the techniques employed, manipulation of nutrient media, optimization of culture conditions, identification of the most effective elicitors and the use of hairy root culture have been given considerable attention.

12.1 Elicitor treatment as strategy to improve production of secondary metabolites

Plants secondary metabolites are majorly produced as a response to protect plants from environmental stresses. The environmental stresses (microbial, physical or chemical factors) leading to an increase in secondary metabolism are known as elicitors. In a broad sense, 'elicitor' for a plant refers to chemical from various sources that can trigger physiological and morphological responses and phytoalexin accumulation associated with plant defense mechanisms. Such interactions usually result in an increase in the production or release of secondary metabolites (Zhao *et al.*, 2005).

The use of elicitors in cell cultures has been developed as one of the main strategies to improve the yield of secondary metabolites wherein elicitation is induced by the addition of trace amounts of elicitors (Radman *et al.*, 2003). Elicitors are signals triggering the formation of secondary metabolites and are classified based on their nature into abiotic or biotic elicitors (Namdeo, 2007).

Elicitors of non-biological origin are called abiotic elicitors, which predominately consist of physical and chemical stress such as UV/gamma radiation extremes of temperature, ethylene, fungicides, antibiotics, salts of heavy metals or high salt concentrations etc.

Other classes of elicitors are biotic elicitors; these are substances with biological origin such as polysaccharides derived from plant cells walls (pectin or cellulose) and micro-organisms (chitin or glucans) and glycoprotein or intracellular proteins which act by activating or inactivating a number of enzymes or ion channels. These classes of elicitors are categorized into two types based on their origin, exogenous and endogenous elicitors. The elicitors which have originated outside the

cell like polysaccharides, polyamines and fatty acids are known as exogenous elicitors whereas endogenous elicitors have originated inside the cell like galacturonide or hepta-β-glucosides etc (Namdeo, 2007). Evidently, in certain circumstances elicitation can be used to obtain better consequence in increasing product yield and has also been found to have commercial potential.

During the process of elicitor study, it should be noted that cells showing failure to elicit necessary secondary metabolites does not necessarily mean that the metabolic pathway cannot be triggered. Inappropriate combination of medium and elicitor, as well as unsuitable concentration of the elicitor could be a cause for unsuccessful elicitation, which indicates that a successful elicitation is a very challenging process requiring intense standardizations (Zhao *et al.*, 2005; Namdeo, 2007). In several studies it has been observed that elicitor treatments performed at the late log phase results in the high biomass yields along with secondary metabolite production as almost all the elicitors, when used at early log phase showed immediate increase in the secondary metabolites while suppressing the biomass leading to overall low productivity. For commercial production of secondary metabolites, apart from the secondary metabolites enhancement, it is equally important to study the signals involved in the process of elicitation, which might help in choosing the appropriate elicitors (Zhao *et al.*, 2005).

12.2 Mechanism of elicitation in plant cells

Several researches have focused mainly on the biotic elicitors, carbohydrate elicitor, in particular, while the effects of abiotic elicitors on over production of secondary metabolites in plants is poorly understood. The elicitation is hypothesized to involve in the key messenger Ca^{2+}, factors affecting cell membrane integrity, inhibition/activation of intracellular pathways and changes in osmotic pressure by acting stress agent (Radman *et al.*, 2003). The primary reactions upon elicitation with a biotic elicitor are the recognition of the elicitor and it's binding to a specific receptor protein on the plasma membrane and the next step, inhibition of plasma membrane ATPase that reduces the proton electrochemical gradient across this membrane (Dörnenburg and Knorr, 1995).

Plants subjected to stresses (including various elicitors or signal molecules) lead to an accumulation of a variety of secondary metabolites. It is important to study these stress related signal transduction thereby helping in the development of strategies for commercial production of the target compounds by either activation or suppression of certain metabolic pathways making it a powerful tool to investigate pathway regulation based on gene expression.

Upon challenge by the biotic elicitors, plants generally stage an array of defence or stress responses. The recent studies are focused on studying the recognition of elicitation stimulus and the subsequent triggered defence response. Signal transduction of plant defence primarily involves the host (cell) recognition to elicitors which initiates the early signaling events such as protein phosphorylation or dephosphorylation, changes in ion fluxes and oxidative burst. These processes stimulate the subsequent transcriptional activation of plant defence genes, which involves activation of enzymes such as glutathione-S-transferases (GST), phenyl ammonium lyase (PAL), chalcone synthase (CHS) etc., biosynthesis of endogenous secondary signals such as salicylic acid etc. and activation of NADPH oxidase leading to complex generation of reactive oxygen species (ROS) such as O_2^-, H_2O_2 and thereby altering the redox status of plant cells affecting the defense signaling. ROS have been found to be generated after any elicitation process. Plants have evolved efficient antioxidant systems to scavenge ROS (Fig. 2.4). The induction/suppression of antioxidant activities provides evidence for occurrence of oxidative burst and variation of secondary metabolite accumulation (Yang *et al.,* 1997).The interaction of elicitors with cellular receptors and subsequent transcriptional and post translational activation of transcription factors thereon lead to the induction of defense genes (Zhu *et al.*, 1996). In addition to eliciting primary defense responses, elicitor signals may be amplified through the generation of secondary plant signaling molecules such as salicylic acid (SA) (Durner *et al.,* 1997). Elicitors and secondary endogenous signals generally activate a diverse array of plant defense and protector genes, whose products include glutathione S-transferases (GST), peroxidises, cell wall proteins, proteinase (PR) proteins and phytoalexin biosynthetic enzymes, such as phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) (Hammond-Kosack and Jones, 1996). Phytoalexins are low-molecular weight, antimicrobial compounds (e.g.,

phenylpropanoids, terpenoids, etc), whose synthesis is induced following biotic elicitation. Furthermore, signalling components such as G proteins, NADPH oxidase, H_2O_2, SA, mitogen-activated protein kinases (MAPK) and Myb transcription factors have been found to participate in several plant defense responses (Fig. 2.5). The appearance of cell death and production of ethylene also play a regulatory role in the elicitor induced defence responses of plants (Yang *et al.*, 1997).

12.3 Elicitors treatment and *in vitro* production of plumbagin

Elicitors have been shown to be effective strategy to achieve and increase production of secondary compounds (Dörnenburg and Knorr, 1995). Elicitors have received wide acceptance because of its ability to improve productivity of the plant cell and organ culture (Zhao *et al.*, 2005).

In general, in vitro plant cell and organ cultures for the production of different secondary metabolites have limited success due to their low yields for commercial application (Buitelaar and Tramper, 1992; Vasconsuelo and Boland, 2007). In addition to the optimization of culture conditions (e.g., medium salt bases, sucrose concentration and pH), strain improvement, and the addition of biosynthetic precursors, the treatment of plant cell and organ cultures with elicitors has been shown to be an effective strategy to increase production of secondary metabolites (Dörnenburg and Knorr, 1995). The increased production, through elicitation, of the secondary metabolites from plant cell cultures has open up a new area of research which would have important economical benefits for industry (Radman *et al.*, 2003).

The effectiveness of elicitation is depended on a complex interaction between the elicitor and the plant cell. However, some of the main factors affected this interaction and thereby the elicitation response such as elicitor specificity, elicitor concentration, treatment interval and culture conditions (growth stage, medium composition and light), affected the secondary metabolite production in different plant species. Several types of products related with naphthoquinones have been successfully elevated by elicitation as shown in Table 2.2.

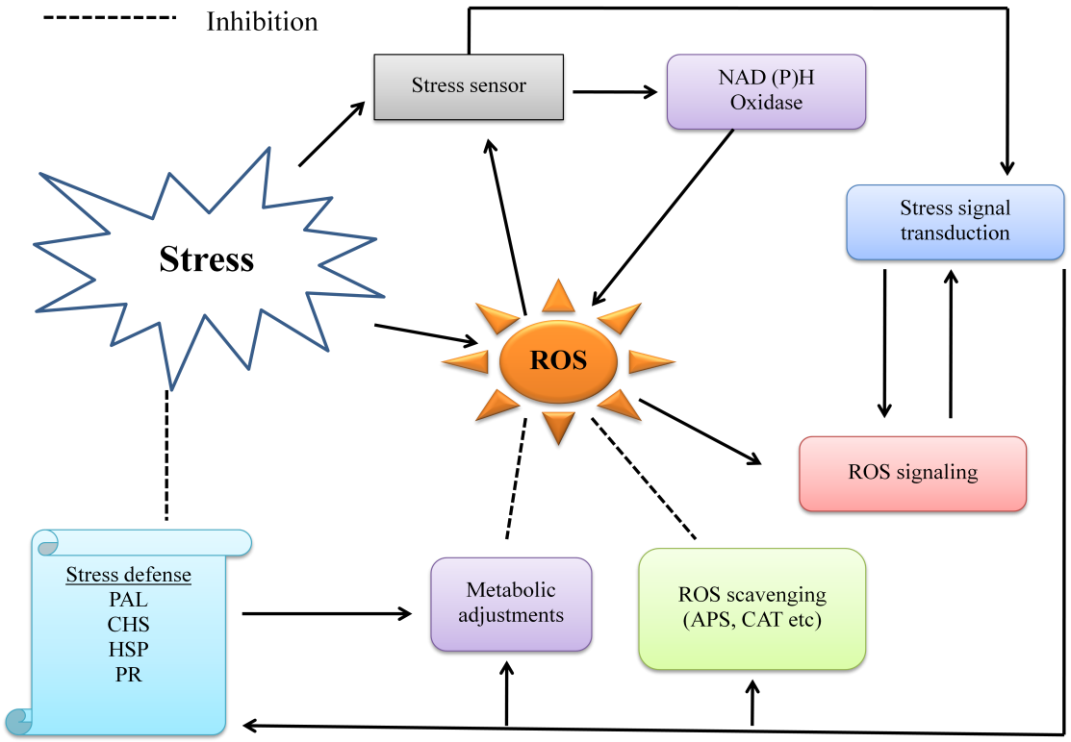


Figure 2.4 Mechanism of ROS production and scavenging during biotic or abiotic stresses. Ascorbate peroxidase (APX) and catalase (CAT) are some of the key ROS scavenging enzymes of plants and heat shock proteins (HSPs); pathogenesis related proteins (PR); phenylaanine ammonia-lyaze (PAL), chalcone synthase (CHS); nicotinamide adenine dinucleotide phosphate-oxidase (NAD(P)H oxidase) are some of the important stress related genes. Source: (Shilpa *et al.*, 2010).

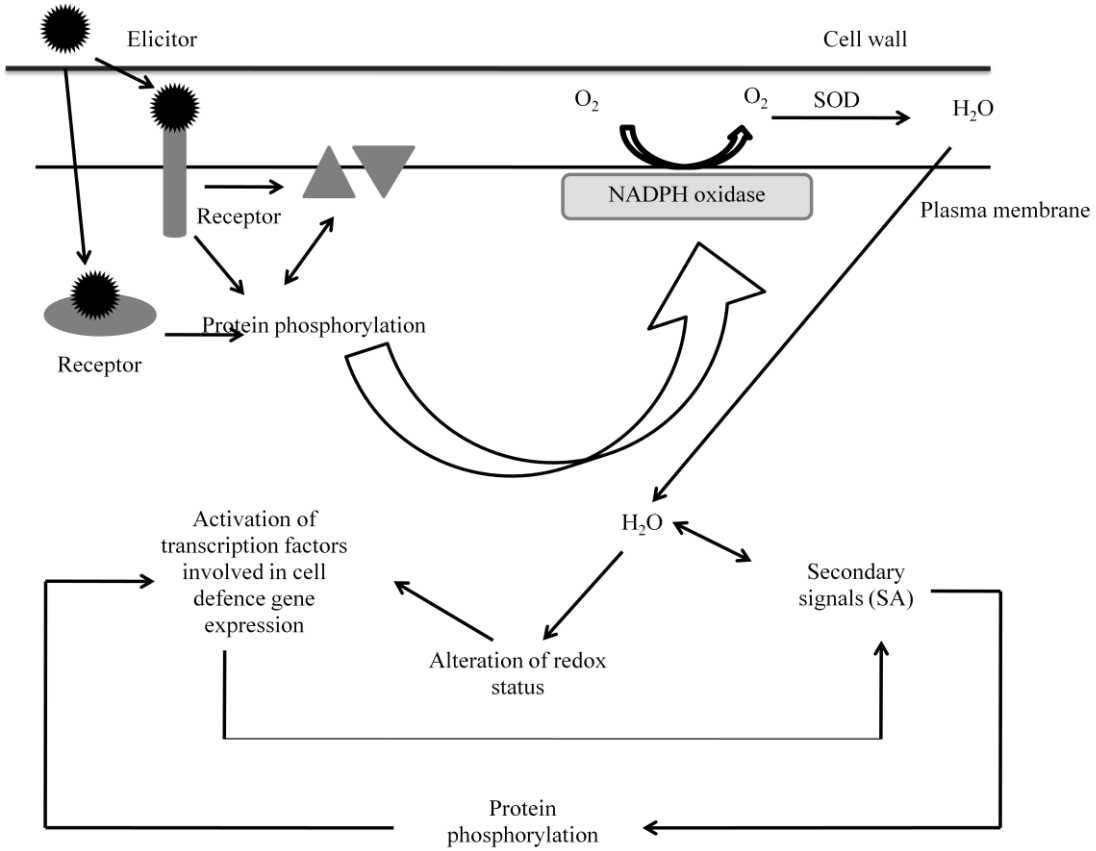


Figure 2.5 Signal transduction involved in plant defense responses. Cell recognizes elicitors and initiates early signaling events. Subsequent transcriptional and posttranslational activation of transcription factors leads to induction of plant defense genes and biosynthesis of endogenous secondary signals. Additionally, the activated NADPH oxidase complex generates reactive oxygen species (ROS), which alter the redox status of plant cells and affect defense signaling. Source: (Shilpa *et al.*, 2010).

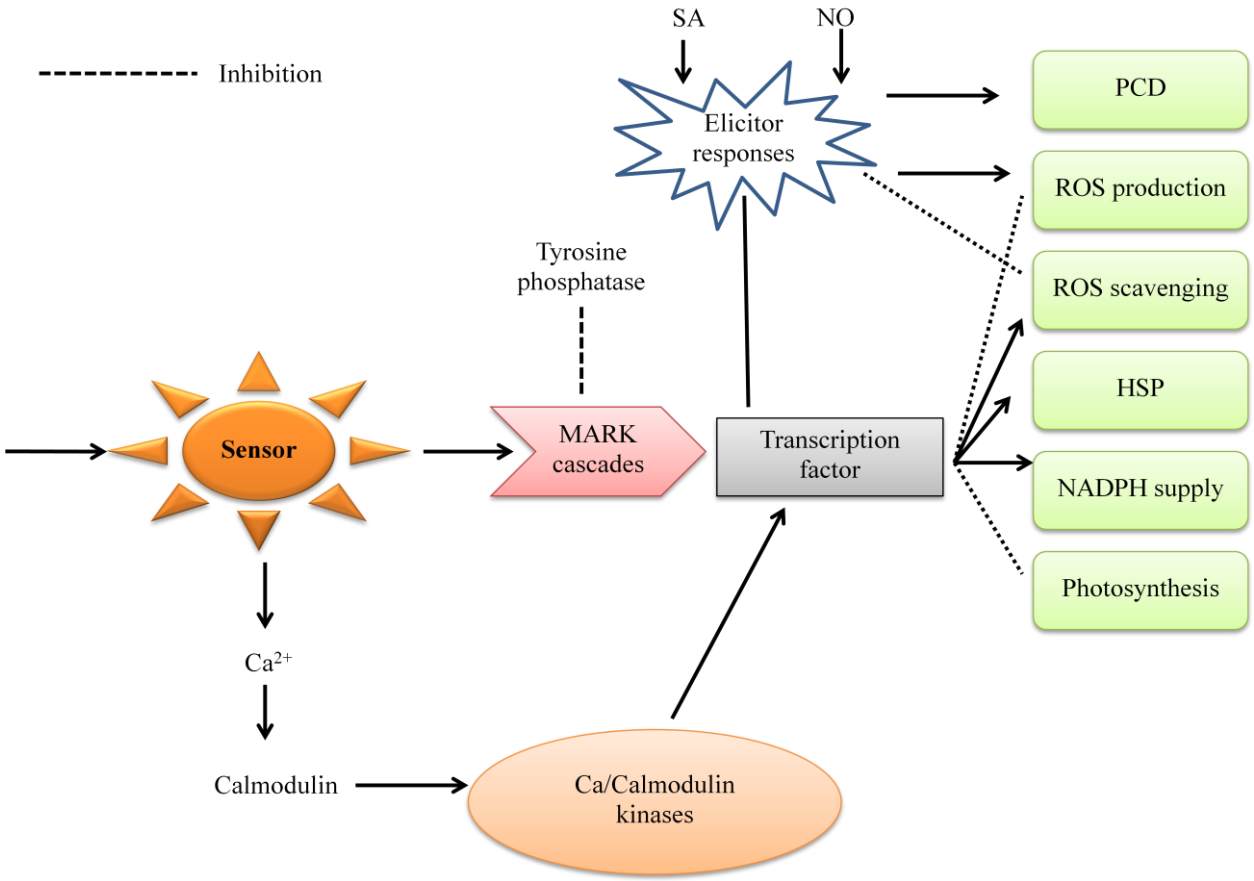


Figure 2.6 The signaling pathway activated in plants in response to external application of oxidants, SA, salicylic acid, NO, nitric oxide, H_2O_2, hydrogen peroxide, PCD, programmed cell death; HSP (heat shock proteins), MAPK (mitogen-activated protein kinase). Source: (Shilpa *et al.*, 2010)25

Table 2.2 Example of naphthoquinone production by elicitation techniques

Plant	Elicitor	Naphthoquinone	Culture type	References
Arnebia euchroma	Fungi	Shikonin	Cell suspension	(Fu and Lu, 1999)
Drosera capensis	Salicylic acid and Jasmonic acid	7-Methyljuglone	Root	(Ziaratnia *et al.*, 2009)
Lithospermum erythrorhizon	Methyl jasmonate	Shikonin	Cell suspension	(Yazaki *et al.*, 1997)
L. erythrorhizon	Fungi	Shikonin	Cell Suspension	(Kim and Chang, 1990)
Plumbago rosea	Chitosan, & yeast extract	Plumbagin	Cell suspension	(Komaraiah *et al.*, 2002)
Impatiens balsamina	Methyl jasmonate and Fungi	Lawsone	Root	(Sakunphueak and Panichayupakaranant, 2010)

Table 2.3 Studies on *in vitro* production of plumbagin

Plant	Techniques	Culture type	Content	References
Plumbago indica	Immobilization	Cell suspension	92.13 mg/g DW	(Komaraiah et al., 2003)
	Acetylsalicylic acid and ammonium	Somatic embryogenesis	12 mg/g DW	(Komaraiah et al., 2004)
P. rosea	Medium Manipulation	Cell suspension	4.5 mg/g DW	(Komaraiah et al., 2001)
	Chitosan	Cell suspension	28.92 mg/g DW	(Komaraiah et al., 2002)
	Medium Manipulation	Root	1.29 mg/g DW	(Panichayupakaranant and Tewtrakul, 2002)
Drosera indica L	Chitosan, Methyl Jasmonate and yeast extract	Whole plant	2.69 ± 0.03 mg/g DW	(Juengwatanatrakul et al., 2011)
D. burmanii	Methyl jasmonate, Chitosan and yeast extract	Shoot	1.5 ± 0.1 mg/g DW	(Putalun et al., 2010)
Drosophllum lusitanicum	Medium Manipulation and Chitin	Cell suspension	3.5 % FW; 1.5 g/L FW	(Nahálka et al., 1996; Nahálka et al., 1998)
Dionea muscipula and Drosera binata	Medium Manipulation	Cell suspension	5.3 % DW and 1.4 % DW	(Hook, 2001)

In recent years, various plant cell culture systems were exploited for the enhancement of secondary metabolites production. Elicitation of secondary metabolites was reported most successful in cell and organ cultures. Many studies

have focused on the production of plumbagin using *in vitro* culture techniques with the use of strategies like medium manipulation and elicitation, which offer an alternative for the production of such pharmaceutically important compounds. Some examples are show in the Table 2.3.

According to recent studies on investigation of plumbagin content on different growing systems using roots of *P. indica*; a) conventionally field grown, b) tissue cultured field growth, c) hydroponically grown plants and d) *in vitro* developed callus from leaf explants. Among the different growing systems of *P. indica*, maximum plumbagin content was observed in the roots of tissue cultured field grown plants (1.80 ± 0.25 g/100 DW) followed by the roots of conventionally field grown (1.33 ± 0.15 g/100 DW), hydroponically grown plants (1.08 ± 0.01 g/100 DW) and callus samples (0.26 ± 0.02 g/100 DW) (Lenora *et al.,* 2012).

Production of plumbagin through suspension culture, immobilization and elicitation has been reported in *P. indica*. Cell cultures of *P. indica* were treated with the elicitors prepared from the fungi (*Aspergillus niger* and *Rhizopus oryzae*), bacteria (*Bacillus subtilis* and *Pseudomonas aeruginosa*), yeast extract and chitosan to induce and enhance the synthesis of plumbagin. Elicitation of plumbagin production in chitosan treated cells was 6.71-fold higher compared to control cells. The treatment of cells with *A. niger*, *R. oryzae* and yeast elicitors resulted in 2-3 fold more plumbagin over control cells. Bacterial elicitors did not show much (<2-fold) influence on plumbagin accumulation (Komaraiah *et al.*, 2002).

In addition, cell cultures of *P. indica* were immobilized in calcium alginate and cultured in MS medium containing 10 mM $CaCl_2$ for the production of plumbagin. Immobilization in calcium alginate enhanced the production of plumbagin by 3, 2 and 1 folds compared to that of control, un-crossed linked alginate and $CaCl_2$ treated cells respectively. Addition of 200 mg/L chitosan as an elicitor to the immobilized cells resulted in eight and two folds higher accumulation of plumbagin over control and immobilized cells. Sucrose utilization rate of the cells was higher when cells were subjected to in situ product removed from the medium. Cells subjected to combined treatments of chitosan, immobilization and in situ extraction showed a synergistic effect and yielded 92. 13 mg/g DCW of plumbagin which is 21,

5.7, 2.5 times higher than control, immobilized, immobilized and elicited cells respectively (Komaraiah *et al.*, 2003).

Furthermore, acetylsalicylic acid and ammonium-induced somatic embryogenesis and enhanced plumbagin production in suspension cultures of *P. indica* was studied. It was found out, optimal embryogenic responses per culture was observed in MS medium containing a combination of ASA (8.32 µM) and IAA (5.06 µM), but NAA and IBA individually didn't induce somatic embryogenesis. Increase in the concentration of ammonium enhanced the number of embryos formed per culture. Accumulation of plumbagin was three times higher in embryogenic compared to non-embryogenic suspensions (Komaraiah *et al.*, 2004).

Assessment of plumbagin production through untransformed root culture with medium manipulation and selection of high yielding plants has been demonstrated in *P. indica*. The production of plumbagin, determined by TLC-densitometry was higher [0.016 ± 0.0030% DW] in cultured roots obtained from B5 supplemented with 1.0 mg/L NAA and 0.1 mg/L kinetin. Plant selection increased the plumbagin production to 0.129 ± 0.0139% DW, while variation of sucrose and nitrogen (as $(NH_4)_2\ SO_4$) concentration in B5 media slightly increased the plumbagin synthesis to 0.023 ± 0.0017 and 0.020 ± 0.0015% DW, respectively (Panichayupakaranant and Tewtrakul, 2002).

We have seen reports of plumbagin accumulation mainly in the Plumbaginaceae family, but reports shows availability of plumbagin in different family such as Droceraceae family. Influence of elicitors on plumbagin accumulation in *Drosera indica* whole plant culture was investigated. Yeast extract (0.5 mg/mL) was the most efficient to enhanced plumbagin production (2.69±0.03 mg/g DW) by 5.4-fold over control plant (0.50 ± 0.01 mg/g DW) (Juengwatanatrakul *et al.*, 2011). In addition, methyl jasmonate, 50 µM, 0.5 mg yeast extract/L and 100 mg chitosan/L stimulated plumbagin production in *Drosera burmanii* whole plant cultures after 6 days of elicitation. Yeast extract (0.5 mg/L) was the most efficient enhancing plumbagin production in roots of *D. burmanii* to 8.8 ± 0.5 mg/g DW that was 3.5-fold higher than control plants (Putalun *et al.*, 2010).

The callus culture of *Drosophyllum lusitanicum* derived from and kept on a Murashige and Skoog (1962) medium accumulated plumbagin 0.08% of

fresh weight. It was found that, at optimal conditions, the plant suspension culture of *Drosophyllum lusitanicum* contained 3.5% FW of plumbagin corresponding to production of 1.5 g l^{-1} per fortnight which is 10 times higher than in the mother plant (Nahálka *et al.*, 1996). In addition, Polysaccharides (chitin/pectin) that are involved in the interactions between plants and microorganisms were applied to the cultured cells of *Drosophyllum lusitanicum*. In the case of chitin addition, elicitation and crystallization of plumbagin in the medium were observed. *N*-Acetylchitooligosaccharides smaller than heptamers [$(GlcNAc)_n$ (n<7)] elicited the biosynthesis of plumbagin but did not increase the hypersensitive response (HR). On the other hand, carboxymethylchitin (DP~200) led to the accumulation of plumbagin in cells and to HR death as well as to the lysis of the cells and release of plumbagin into the medium. The response of cultured cells to the *N*-Acetylchitosaccharides varied depending on the chemo/physiological conditions of the cells. Addition of pectin (1g/l) resulted in enhanced HR and decreased biosynthesis of plumbagin (Nahálka *et al.*, 1998).

13. Gamma ray irradiation and Secondary metabolites production

Gamma ray irradiation as a phytosanitary treatment of food and herbal materials is increasingly recognized throughout the world. It improves the hygienic quality of various foods and herbal materials and reduces the losses due to microbial contamination and insect damages (IAEA, 1992; Farkas, 1998). Such facts of gamma ray irradiation find it promising and many countries have adopted as a way to ensure the hygienic quality of dehydrated foods. The international safe dose clearance is up to 10 KGy, though some countries have increased this level to 30 KGy without any harmful effects been observed (Pérez *et al.*, 2007).

There is a growing scientific interest in the influence of irradiation process on antioxidant activity and the compounds responsible for the activity. Several studies on plants materials showed that gamma ray irradiation does maintain or enhanced antioxidant properties. However, some studies have shown that gamma irradiation decreased the antioxidant properties in plants materials. Gamma ray irradiation

increased the extraction yield and total phenolic content as well as enhancing the free radical scavenging activity (Khattak *et al.*, 2008).

Gamma ray irradiation creates genetic variability in plants which can be screened for desirable characteristics. Gamma ray is a mutagen with high radiation energy, which can lead to damage to covalent bonding, hydrogen bonding and or other molecular bonding of biomolecules in the cell and thus causing chromosomes damage, gene damage and eventually cell death (Vonarx *et al.*, 1998). Choice of suitable dose for mutant generation has to be compromised between the mutagenic effects and damaging effects of the irradiation.

Gamma ray irradiation was reported to induce oxidative stress with overproduction of reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxides, which react rapidly with almost all structural and functional organic molecules including proteins, lipids and nucleic acids causing disturbance of cellular metabolism (Salter and Hewitt, 1992). Free radicals generated in plants during irradiation may act as stress signals and may trigger stress responses in plants.

Moreover, various research works have been done to study the effect of gamma irradiation in the plants in respect of its morphological, physical and biological study. The yield increase of secondary metabolite production was examined in plant cell cultures with the use of relatively low to high doses gamma ray irradiation. Such as, Chung and his group (2006) had studied on cell suspension culture of *Lithospermum erythrorhizon* exposed to gamma ray irradiation with 2, 16 & 32 Gy. The gamma ray irradiation significantly stimulated the shikonin biosynthesis of the cells and increased the total shikonin yields by 400% at 16 Gy, and by only 240% & 180% at 2 & 32 Gy, respectively. Moreover, one of the key enzymes for the shikonin biosynthesis of cells, *p*-hydroxylbenzoic acid (PHB) geranyltransferase, was found to be stimulated by the gamma-radiation treatments. The activity of PHB geranyltransferase was increased at 2 and 16 Gy with a negligible change at 32 Gy. In contrast, the activity of PHB glucosyltransferase was slightly changed at all doses of gamma radiation compared with the control cells. Therefore, the increase in PHB geranyltransferase activity leads to the accumulation of secondary metabolites such as

a shikonin, which may contribute to plant defense against the stresses induced by gamma irradiation.

Hossam and his group (2011) had studied on effect of low doses of gamma irradiation (0, 5, 10, 15 & 20 Gy) on oxidation stress and secondary metabolites production of *Rosmarinus officinalis*. It was found that a highly metabolic modification of chemical constituents and various antioxidant defense enzymes (Ascorbate peroxidase, Catalase etc), which gradually increased in response to radiation doses, while reduced glutathione (GSH), ascorbic acid (AsA) contents, total soluble protein, total soluble amino acids, total soluble sugars and PAL activity positively correlated with the increased doses. On the other hands the high irradiation levels significantly increased the accumulation of various oxidative burst (MDA, H_2O_2 and O_2-). Meanwhile, higher doses of gamma ray irradiation positively enhanced secondary product accumulation of total phenols and total flavonoids in rosemary callus culture.

In addition, Sumira and her group (2010) studied on the effect of various dose of gamma ray irradiation in seeds of *Psoralea corylifolia*. Seeds were exposed to gamma ray radiation with doses of 2.5, 5, 10, 15 & 20 KGy at dose rate of 1.65 $KGyh^{-1}$. It was found that *P. corylifolia* showed highest concentration in seeds (7.56%) at 20 KGy and lowest in control roots (0.23%). The results showed in depth development stimulation and enhancement of secondary metabolite in *P. corylifolia* following low and high dose treatment respectively depicting the potential of gamma rays in plant biotechnology and metabolomics.

Furthermore, Bassam and Rana (2011) studied the improvement of Caper (*Capparis spinosa* L.) propagation using *in vitro* culture and gamma ray irradiation. The effect of gamma ray irradiation on the growth of caper shoots *in vitro* was also studied. A 10 Gy dose of gamma ray irradiation stimulated growth of shoots up to 200% and increased shoot rooting percentage from 75 to 100%.

CHAPTER 3

MATERIALS AND METHODS

1. Materials

1.1 Plant materials

P. indica young leaves were collected from the botanical garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The voucher specimen (Specimen No. SKP 148 16 09 01) was identified by Associate Professor Pharkphoom Panichayupakaranant and deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University. The leaves were washed thoroughly with distilled water to remove any debris, and used immediately.

1.2 Chemicals

Standard plumbagin was purchased from sigma (USA). Elliptinone and 3,3'-biplumbagin used were previously purified extract of Kaewbumrung and Panichayupakaranant (2011). Chemicals used in this study are shown in Table 3.1.

Table 3.1 List of chemicals and reagent

Chemical	Company, Country
1-Naphthylacetic acid (NAA)	Fluka, Switzerland
6-Benzylaminopurine (BA)	Fluka, Switzerland
Ammonium sulfate ($(NH_4)_2. SO_4$)	Merck, Germany
Boric acid (H_3BO_3)	Fisher Scientific, England
Calcium chloride ($CaCl_2. 2H_2O$)	Merck, Germany
Cobalt chloride ($CoCl_2. 6H_2O$)	Fluka, Switzerland
Cupric sulfate ($CuSO_4. 5H_2O$)	Merck, Germany
Ethanol (95%v/v)	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Ferrous sulfate ($FeSO_4. 7H_2O$)	Fisher Scientific, England
Manganese sulfate ($MnSO_4. H_2O$)	Fluka, Switzerland
Magnesium sulfate ($MgSO_4. 7H_2O$)	APS Ajax Finechem, Australia
Methanol HPLC grade	Lab-scan Asia Co., Ltd., Bangkok, Thailand.
Myo-inositol	VWR International Ltd, England
Nicotinic acid	Fluka, Switzerland
Plant agar	Duchefa Biochemic, The Netherlands
Potassium Iodine (KI)	Merck, Germany
Potassium nitrate (KNO_3)	VWR International Ltd, England
Pyridoxine hydrochloride	Fluka, Switzerland
Sodium hypochlorite (Clorox®Regular bleach)	Clorox Company, USA
Sodium phosphate ($NaH_2PO_4. H_2O$)	Sigma, Germany
Sucrose	MITRPOL, Thailand
Zinc Sulfate ($ZnSO_4. 7H_2O$)	Fluka, Switzerland

3.1.3 Instrumentations

Table 3.2 List of equipments and instruments

Instrumentation	Company, Country
Autoclave machine, Model HA-3D	Hirayama, Japan
Centrifuge, Kubota 5922	Kubota corporation, Japan
Hot air oven, Memmert	Schwubuch, Germay
Hot plate and stirrer, CORNING	Fisher Scientific, USA
HPLC, Binary pump 1525	Waters Corporation, USA
Photo diode array detector, Waters 2998	Waters Corporation, USA
Auto sampler, Waters 2707	Waters Corporation, Netherland
HPLC column, Phenomenex® ODS column	Tosho Bioscience, Japan
Laminar air flow cabinet, HT-122	Holten, Denmark
Micropipette, ACURA 825	Orion Research, Switzerland
Hot air oven, DIN 12880-KI	Memmert, Germany
pH meter, Model 710 A	Thermo Electric company, USA
Rotary evaporator, N 1000	EYELA, Japan
Shaker, Innova 2300	Illinois, USA
Ultrasonic bath, S 100H	Crest Ultrasonic Corporation, USA
Quanta Scanning Electron Microscope (SEM)	FEI, Czech Republic

2. Methods

2.1 Preparation of *P. indica* leaf explants

The young leaves of *P. indica* were washed with running tap water for 2 h and rinsed 3 times with distilled water. The leaves were then dipped in 70% v/v ethanol for 10 s and subsequently soaked in 20% v/v Clorox® solution for 15 min. After that, the sterile leaves were rinsed 3 times with sterile distilled water and cut with a sharp scalpel. The explants were then transferred to solid media under aseptic conditions.

Table 3.3 Stock solutions for Gamborg's B5 medium (Gamborg *et al*., 1968)

Stock 1a Macronutrients (x20)	**g/500 mL**
KNO_3	25.00
$MgSO_4.7H_2O$	2.50
$NaH_2PO_4.\ H_2O$	1.50
$(NH_4)_2SO_4$	1.34
Stock 1b Macronutrients (x10^3)	**g/100 mL**
$CaCl_2.2H_20$	15.00
Stock 2 Micronutrients (x10^3)	**g/100 mL**
H_2BO_3	0.30
$MnSO_4.\ H_2O$	1.00
$ZnSO_4.7\ H_2O$	0.20
$NaMo_4.2\ H_2O$	0.025
$CuSO_4.5H_2O$	0.0025
$CoCl_2.6H_2O$	0.0025
Stock 3 KI (x10^3)	**g/100 mL**
KI	0.075
Stock 4 (Fe-EDTA)	**g/100 mL**
$FeSO_4.7H_2O$	2.78
Na_2EDTA	3.72
Stock 5 Vitamins (x100)	**g/100 mL**
Thaimine hydrochloride	1.00
Pyridoxine hydrochloride	0.10
Nicotinic acid	0.10
Myo-Inositol	10.00
Plant growth regulators	**g/100 mL**
NAA stock solution (100 mg/L)	0.01

2.2 Preparation of Gamborg's B5 media

To prepare B5 medium the stock solutions were prepared as shown in Table 3.3. Stock 1a (50 mL), 1b (1 mL), 2 (1 mL), 3 (1 mL), 4 (5 mL) and 5 (1 mL) were combined together. The plant growth regulators were added as needed and adjusted the volume to 1000 mL. The pH of the medium was adjusted to 5.5 with 1N NaOH or 1N HCL. Plant agar (8 g/L) was used to solidify the medium.

2.3 Establishment of *P. indica* root cultures

The young leaf explants of *P. indica* were cultured on solid Gamborg B5 medium (Gamborg *et al.,* 1968) supplemented with 0.1 mg/L of α-naphthalene acetic acid (NAA), 20 g/L sucrose, 0.8 % (w/v) agar, and incubated at 25° ± 2°C under dark conditions. The root formation and morphological appearances in each flask were observed every day. The obtained roots (2 g fresh weight) were transferred to 250 mL Erlenmeyer flasks containing 50 mL of liquid B5 medium supplemented with the same hormonal composition. The root cultures were incubated on a rotary shaker (80 rpm), at 25 ± 2°C under dark conditions. Maintenance of the cultures was carried out by periodic subculture (4-week intervals).

2.4 Extraction of plumbagin from the root cultures

The root cultures were harvested using vacuum filtration and dried at 50 °C in a hot air oven. The dried roots were ground to a fine powder using a grinder. The plumbagin was extracted from dried root powder (100 mg) by using ethanol (20 mL) under reflux condition for 20 min and then filtered. The filtrates were evaporated to dryness under reduced pressure (40°C). The residues were reconstituted in methanol and the volume was adjusted to 5 mL (Kaewbumrung and Panichayupakaranant, 2011). Samples were filtered through a 0.45 μm membrane filter and subjected to quantitative HPLC analysis for plumbagin content. The experiment was performed in triplicate.

2.5 Extraction of plumbagin from liquid media

Culture medium (50 ml) was acidified with 1N HCl to pH 2.0, and then partitioned with ethyl acetate (20 ml × 3). The pooled ethyl acetate fractions were then evaporated to dryness under reduced pressure (40°C). The residue was reconstituted in methanol and the volume adjusted to 10 ml (Sakunphueak & Panichayupakaranant, 2010).

2.6 Quantitative HPLC analysis of plumbagin

HPLC analysis was carried out using the method described by Kaewbumrung & Panichayupakaranant (2011). HPLC analysis was carried out using a binary HPLC pump (Waters 1525, USA) equipped with a photodiode array detector (Waters 2998, USA) and autosampler (Waters 2707, Netherland). Separation was achieved at 25 °C on a Phenomenex® ODS column (5 µm, 150 mm × 4.6 mm i.d.). The mobile phase consisted of methanol and 5% aqueous acetic acid (80:20, v/v), and was pumped at a flow rate of 0.85 mL/min. The sample injection volume was 10 µL and quantitative detection was observed by UV spectrum at wavelength 260 nm.

Stock solutions of standard plumbagin (0.5 mg/mL) was prepared in methanol and diluted to provide a series of the standard solutions containing 3.125, 6.25, 12.5, 25, 50, 100 and 200 µg/mL. These solutions were subjected to HPLC analysis, and a calibration curve was constructed for plumbagin by plotting peak areas against the concentration.

2.7 Time course of growth and plumbagin production of *P. indica* root cultures

The root cultures of *P. indica* (3-week old, 2 g) were transferred to fresh B5 liquid medium. The root cultures (5 flasks) had been harvested on the initial day and every 4 days for a period of 36 days. The harvested roots were dried at 50^{o}C for 24 h. The dry weights were recorded. The dried root powders were then subjected

to determination of the plumbagin content by HPLC as described in the section 2.4. The time courses of growth and plumbagin production were constructed by plotting dry biomass (g/flask) and plumbagin content (% w/w) against the culture age (days).

2.8 Elicitation preparation

2.8.1 Gamma ray irradiator

The gamma irradiator used for the elicitation was generated by ^{60}Co (capacity. 381TBg Theratron Phoenix) at the Songklanagarind Hospital, Prince of Songkla University, Hat Yai, Songkhla Thailand. The gamma ray irradiator gave gamma rays at 3.1KGyh^{-1} dose rate at its initial instillation**.**

2.8.2 Radiation dose

The amount of radiation energy that a material absorbed is measured in SI units called “gray (Gy)” One gray is equivalent to one joule per Kg (IAEA, 1990). The Gray replaced the earlier unit, the rad (1 Gy = 100 rad).

2.8.3 Dose rate

The dose rate is the quantity of radiation absorbed per unit time. The dose rate used during the experimentation was based on the following equation and calculations as per (Table 3.4).

$$\boldsymbol{A = A_o e^{-\lambda t}}$$

$\boldsymbol{A}$ = Dose rate during experimentation

$\boldsymbol{A_o}$ = Dose rate at initial instillation

$\boldsymbol{\lambda}$ = Decay constant

$$\boldsymbol{\lambda = In(2) / T\,{}^{1}/_{2}}$$

Where;

$In(2)$ = 0.693

$T\,{}^{1}/_{2}$ = Half life of Co-60

$\boldsymbol{t}$ = Time period (No. of days from initial instillation until date of use)

2.8.4 Irradiation time

The required or the selected radiation dose radiated to the materials for a particular period of time is referred to irradiation time. The irradiation time used in the series of experimentation is shown in Table 3.4. This irradiation time was calculated based on the following equation;

$$Irradaition\ time = \frac{\boldsymbol{D}}{\boldsymbol{DS} \times \boldsymbol{OF} \times \boldsymbol{TMR} \times \boldsymbol{TF} \times \boldsymbol{WF}}$$

Where;

$\boldsymbol{D}$ = Selected or prescribe dose

$\boldsymbol{DS}$ = Dose rate

$\boldsymbol{OF}$ = Output factor (Derived from measurements)

$\boldsymbol{TMR}$ = Tissue Maximum Ratio (Standard protocol)

$\boldsymbol{TF}$ = Tray factor (When tray or block materials are used)

$\boldsymbol{WF}$ = Wedge factor (When wedges are used)

Table 3.4 Gamma ray irradiation condition during dose optimization

Dose (Gy)	Irradiation time (mins)	Dose rate (Gy/mins)
5	6.47	0.77
10	12.94	0.77
15	19.41	0.77
20	25.88	0.77
25	32.35	0.77

Table 3.5 Gamma ray irradiation condition during age optimization

Day	Irradiation time (mins)	Dose rate (Gy/mins)
0	26.2	0.76
5	26.84	0.75
10	26.88	0.74
15	26.92	0.74
20	27	0.74

2.9 Determination of optimum gamma ray dose

The root culture of *P. indica* (3-week old, 2 g) was transferred to fresh B5 liquid media (50 mL). Various gamma ray doses (0, 5, 10, 15, 20 & 25 Gy) selected were irradiated to the 12 day old root cultures (mid-linear phase). Six individual root cultures were used for each dose. After the gamma ray irradiation, the root cultures were further incubated until reaching a harvested period (24-day old). The cultured roots were harvested by vacuum filtration washed with water, dried at 50°C, determined its dry weight, and then powdered, extracted and subjected to quantitative HPLC analysis for plumbagin. An optimum gamma ray dose was selected for further experiment.

2.10 Determination of optimum age of the culture for elicitation

The most appropriate gamma ray dose was selected from earlier experiment. An appropriate age of the root cultures for maximal elicitation was investigated. An appropriate gamma ray dose was treated to 0, 5, 10, 15 and 20 days old root cultures. After the gamma ray irradiation, the root cultures were further incubated until reaching a harvested period (24-day old). The cultured roots were harvested by vacuum filtration, dried at 50°C, determined their dry weights and then powdered, extracted and subjected to quantitative HPLC analysis for plumbagin.

2.11 Determination of naphthoquinones production profile in the *P. indica* root culture

The control and irradiated root cultures were analyzed and studied for the naphthoquinone content. The root extract was subjected to HPLC analysis to determine the content. The protocol for determination was followed as in the previous sections 2.4, 2.5 and 2.6.

2.12 Determination of cell morphology by Scanning Electron microscope (SEM)

The 20 Gy irradiated root cells and the control root cells were subjected to the microscopic study for the examination of the effects of gamma radiation on the morphological structure of the cells. The SEM observation was followed based on the previous reports (Longstreth and Borkhsenious, 2000) with little modification. The cultured roots were harvested by vacuum filtration, dried at 50°C in hot air oven for 12 h. The dried roots were cut into 4-5 mm pieces using razor blade for SEM preparation for cross section view.

The dried root sections were taped to the specimen stubs coated with silver, conductive carbon tape. The tissue was fixed and vacuum infiltrated with 2.5 % glutaraldehyde in 0.1M Phosphate buffer solution, pH 7.4, for 2 h. After washing with buffer, sections were then dehydrated in a graded ethanol series and critically point dried (Polaron, CPD-7501, England) with liquid carbon dioxide. The sections were coated with gold ± palladium in a vacuum evaporator (SPI-Module^{Tm} Sputter Coater, USA), then viewed and photographed on a Quanta 400 SEM (FEI Scanning Electron Microscopy, Czech Republic). Micrographs were digitized and measurements of morphological and the cortex cell dimensions were made on representative transverse sections using ACDSee Pro 5 software (ACD Systems International Inc., USA).

2.13 Determination on stability of elicited root cultures for plumbagin production

The elicited roots cultured with the optimized conditions were further studied for the stability on the growth and plumbagin production based on subculture from the initial elicited root to further 3 generations. The root culture elicited with 20 Gy to a 12 days old root culture was further subculture into a new generation batch after 24 days of complete incubation. It was further carried out for the next three batches of subcultures with harvesting of 3 flasks per batch for both control and elicited root cultures. The root extract was then prepared and subjected to HPLC analysis based on the protocol mentioned in section 2.4, 2.5 and 2.6. The stability studies were based on the biomass and HPLC determination on the plumabgin production.

2.14 Statistical analyses

All experiments were repeated three times. The data [mean ± standard error (SE)] obtained were statistically analyzed by SPSS version 15.0. Data were subjected to analysis of variance, and the means were then compared with one-way ANOVA, using Tukey's test for multiple comparison. The term significant had been used to denote the differences for which $P < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

1. Establishment of *Plumbago indica* root cultures

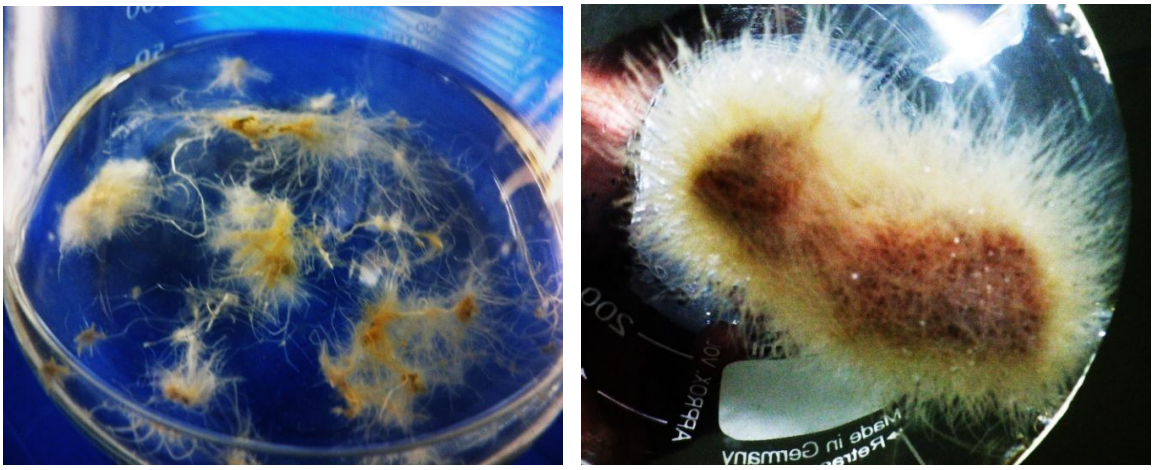
The most exploited sources of plumbagin are the roots of *Plumbago* spp. However, these plants grow slowly and take several years to produce quality roots suitable for use (Kitanow and Pashankov, 1994). Recently, plant tissue culture methods are taken as attractive alternative sources to whole plants for the production of high-value secondary metabolites and improve the productivity (Rates, 2001). In the previous report, we had described an establishment of *P. indica* root cultures, using a selection of plumbagin high yielding plant as an explant for the root initiation (Panichayupakaranant and Tewtrakul, 2002). However, the root cultures still produced low plumbagin level (1.29 mg/g dry weight). Production of plumbagin through suspension culture, immobilization and elicitation has been reported in *P. indica* (Komaraiah *et al.*, 2003). However, there are still several problems in the production of plumbagin by the cell cultures such as the instability and slow growth of the cell lines and low yields of plumbagin. In this study, we report the establishment of *P. indica* root cultures, with increased plumbagin accumulation.

The root cultures of *P. indica* were not established from the root explant, because the survival rate was very low due to a high contamination and slow regeneration rate of the roots. The young leaves of *P. indica* were the most suitable plant material for root initiation due to low microbial contamination and high root producing rate. The root cultures of *P. indica* were therefore established from the young leaf explants (Figure 4.1A). In addition, only the explants that initiated under the dark conditions were capable of producing the root cultures. This result agrees with that report on an establishment of *Rhinacanthus nasutus* (L.) Kurz (Acanthaceae) root cultures that the root formation was inhibited by light (Panichayupakaranant and Meerungrueang, 2010). The roots were regenerated from the leaf explants after 2-week initiation. White puffy roots were formed on the solid B5 medium supplemented

with 0.1 mg/mL NAA within 8 days of culture (Figure 4.1B). The root biomass were increased and maintained by transferring into the liquid B5 medium. The cultured roots in liquid B5 medium appeared as a small aggregation of hairy sharp tiny roots (Figure 4.2A). The physical appearance of the roots turned from white to dark brown puffy roots after several cycles of subcultures (Figure 4.2B).

A **B**

Figure 4.1 Root initiation from the leaf explants of *P. indica* on solid B5 media



A **B**

Figure 4.2 Root cultures of *P. indica* in liquid B5 media

2. Plumbagin production by the root cultures

The HPLC method described previously was used for quantitative analysis of plumbagin because it is simple, specific, precise and accurate (Kaewbumrung and Panichayupakaranant, 2011). The calibration curve of plumbagin were established using the authentic compound (concentration range was 3.12 - 200 µg/mL). Plumbagin exhibited linearity over the evaluated ranges, with the linear equations of Y = 77821X - 136453(R^2 = 0.9997) (Figure 4.3).

Concentration of plumbagin (µg/mL)	Peak area			
	1	2	3	Average
3.125	177268	175600	175519	176129
6.25	373092	374362	374736	374063.3
12.5	772495	774188	776353	774345.3
25	1767385	1775587	1780039	1774337
50	3690043	3755792	3760355	3735397
100	7678931	7659175	7665770	7667959

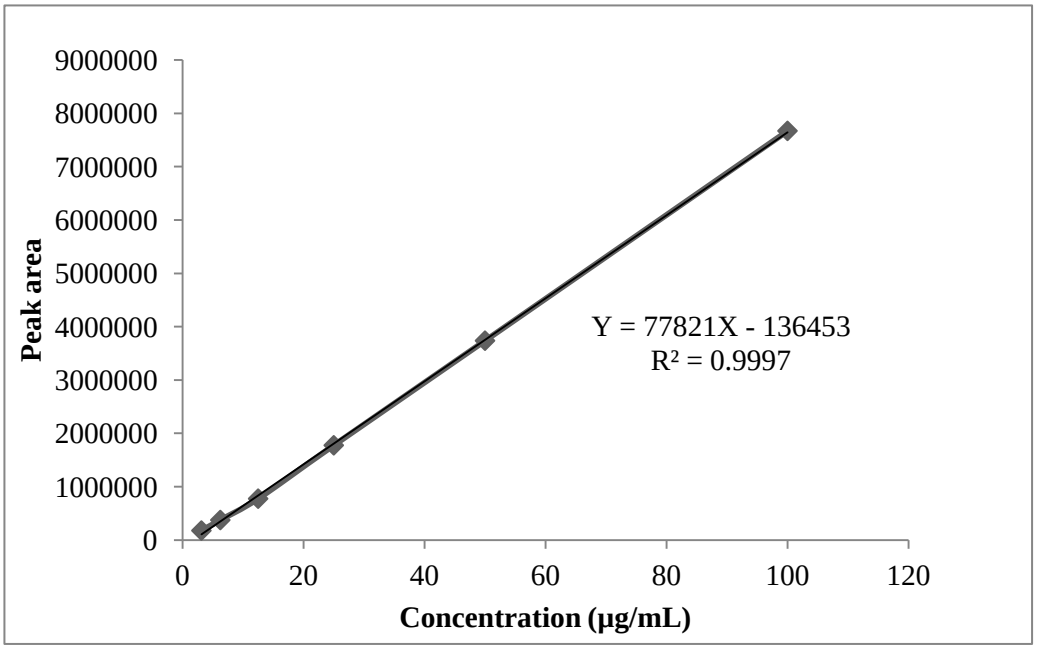


Figure 4.3 Calibration curve of plumbagin

On the basis of HPLC analysis, the root cultures produced plumbagin as the major naphthoquinone (Figure 4.4). The identity of the plumbagin peak in the HPLC chromatogram of the root culture extract was confirmed by the UV absorption spectra produced by photo-diode array detector compared with that of the authentic plumbagin.

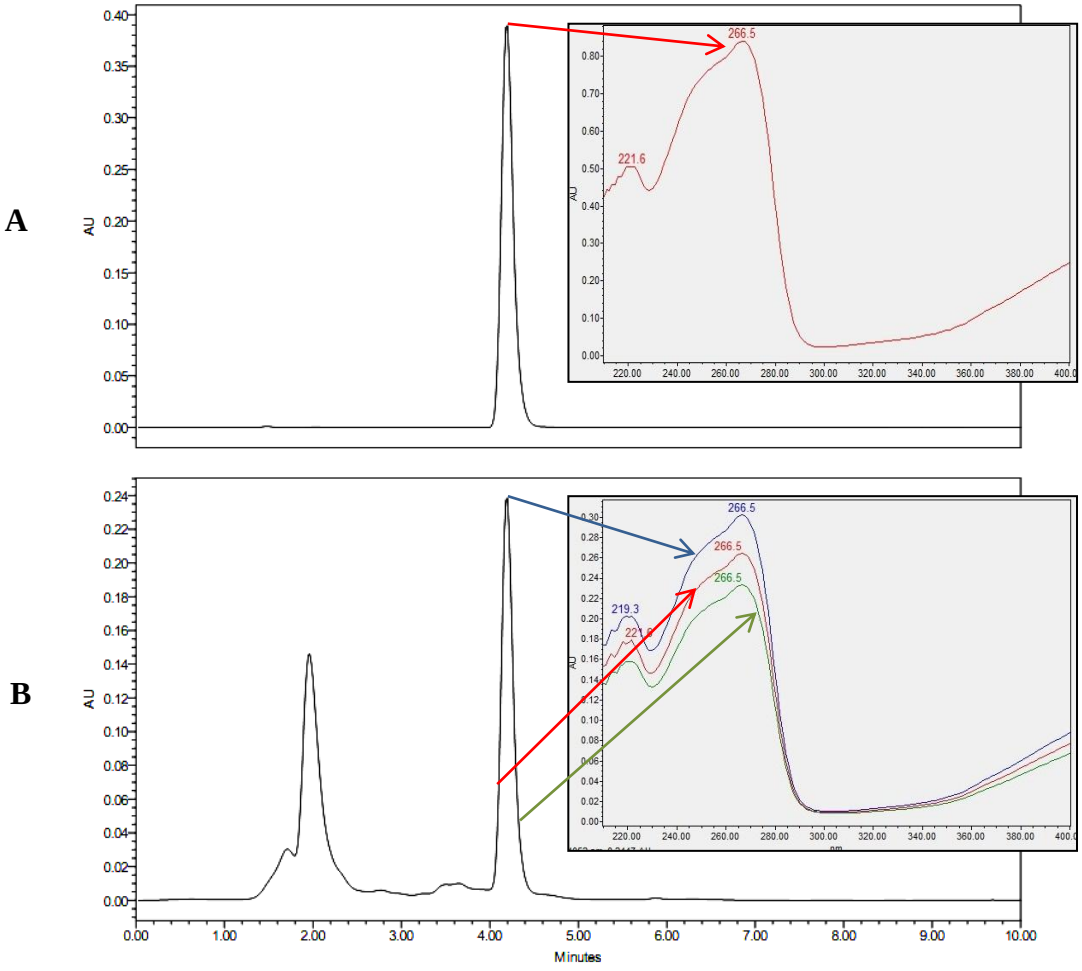


Figure 4.4 HPLC chromatograms of (A) authentic plumbagin and (B) the root culture extract

These root cultures accumulated plumbagin with a higher level (2.21 ± 0.70 mg/g DW) than the previous reports of the root cultures of *P. indica* (1.29 ± 0.14 mg/g DW) (Panichayupakaranant and Tewtrakul, 2002), and the shoot cultures of

Drosera burmanii (1.5 ± 0.1 mg/g DW) (Putalun *et al*., 2010). However, the plumagin level accumulated in the root cultures is still lower than that of the intact roots of 2-years old *P. indica* (8.6 ± 1.9 mg/g DW) (Panichayupakaranant and Tewtrakul, 2002). Although the root cultures produced less plumbagin level than the intact roots, the growing time is shorter when compared to field growing plants.

3. Time courses of growth and plumbagin production of *P. indica* root cultures

In this study, time courses of growth and plumbagin production of the root cultures were described. The root cultures spent 24 days for their growth cycle with a very short lag phase (Figure 4.5). There was a continuous increase in the biomass during 16 days. Thereafter, the dry weight of the biomass decline relatively indicating that the cultured reached to a stationary phase at day 20, and gave the highest biomass (0.39 g/flask), which was 3 times the biomass of the inoculated roots.

During the 24-day period of the culture growth, it was found that plumbagin was initially accumulated in the linear phase (after day 12) and actively biosynthesized until reaching the stationary phase (Figure 4.5). This observation indicated that the biosynthesis of plumbagin took place in-line to the growth of the root cultures. The highest content of plumbagin was observed at day 20 (0.86 mg/flask DW); after that the production rate began to slow down. This suggested that the suitable period of the root cultures is about 3-weeks old.

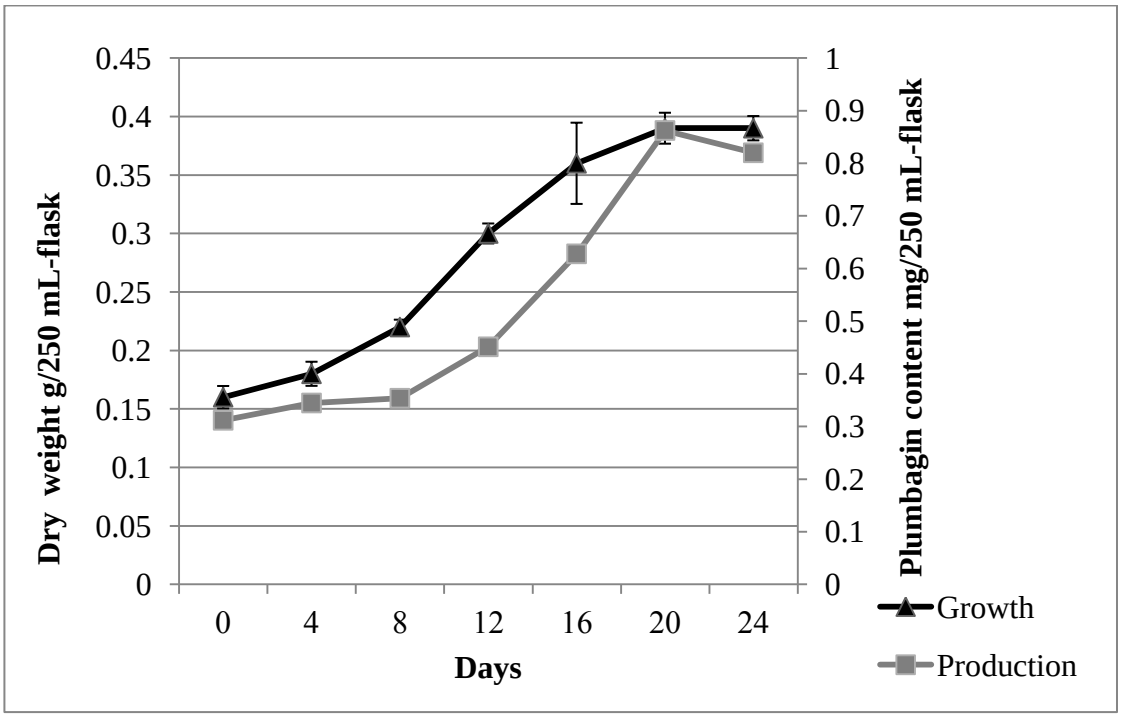


Figure 4.5 Time courses of growth and plumbagin production in *P. indica* root cultures

4. Effects of different doses of gamma rays on plumbagin production

Recently, it has been reported that low doses of gamma ray significantly stimulated secondary metabolite production in several plant tissue cultures. An important factor which affects an increase in secondary metabolite production by gamma ray is the dose given to a plant tissue culture. Moreover, ionizing radiation have been successful in inducing genetic variability in many plant species (Kim *et al*., 2005), there has been no information in the literature on using this technique to induce plumbagin variation in *P. indica* root cultures.

The optimal dose of gamma ray was therefore determined based on the effect of plumbagin productivity, and the data used for subsequent experiment. Gamma ray irradiation with various doses on the 12-day old root cultures (in a linear growth phase) and harvested at 24 days of culture, showed that there was almost no significant difference in growth patterns between the treated and untreated groups. This might be due to the treatments of root cultures during the linear growth phase.

On the basis of HPLC analysis, only plumbagin was the naphthoquinone produced by both treated and untreated root cultures. The effect of all doses treated was positive on the production of plumbagin (Figure 4.6). This observation is in line with the previous report on effect of low dose of gamma ray irradiation on shikonin production by cell suspension cultures of *L. erythrorhizon* (Chung *et al*., 2006), and on flavonoids production of *R. officinalis* callus cultures (Hossam *et al*., 2011). A treatment of a low dose gamma irradiation also affected on the secondary metabolism of *Artemisia annua* (Koobkokkruad *et al*., 2008). Such, apparent variation of plumbagin content in the irradiated root cultures might be the mutagenic effects of gamma rays to the gene involved in biosynthetic pathway of plumbagin as seen previously in suspension culture of *L. erythrorhizon* (Chung *et al*., 2006) and plantlets of *A. annua* (Koobkokkruad *et al*., 2008).

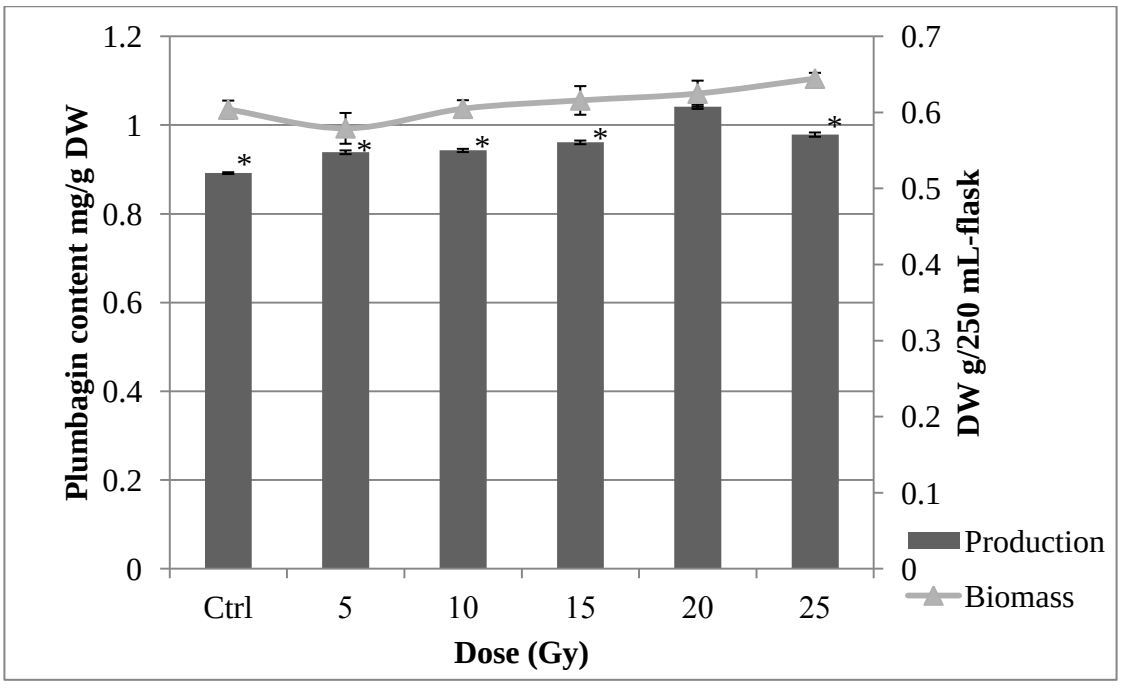


Figure 4.6 Effect of different dose (Gy) on growth and plumbagin production of *P. indica* root cultures (gamma ray treatment on 12-day old root cultures). CTRL is the untreated group. Data are mean ± SE of n = 3. *significant difference (P<0.05) when compared with the 20 Gy groups.

Although all treated groups were capable of increasing plumbagin production, the treated dose at 20 Gy exhibited significantly increased plumbagin production when compared to all groups. Thus, the optimum dose of gamma ray for increased plumbagin production by *P. indica* root cultures was 20 Gy. Gamma ray at 20 Gy was therefore used for further study on the effect of culture age on plumbagin production by the root cultures.

5. Effects of culture age

According to the time course of growth and plumbagin production of *P. indica*, plumbagin was initially accumulated in a linear phase (day 12) and actively biosynthesized until reaching the stationary phase. Cells at different stages of growth have different levels of mRNA and proteins, and elicitor treatment on different growth phases may yield varied responses in terms of cell growth and secondary metabolite production (Chong *et al*., 2005). In this study, the ages of root cultures were varied as an initial day (day 0), 5, 10, 15 & 20 days. The root cultures were exposed to the gamma radiation (20 Gy), the roots then harvested after 24-day old and subjected to plumbagin content determination. An increase of plumbagin content was observed in all treatment ages of cultures. The appropriate age of root cultures for maximum production of plumbagin (1.64 mg/g DW) was found to be 10 days, which was 2.94-fold higher than the level in the control (0.56 mg/g DW) (Figure 4.7). However, treatment of 5-day old root cultures resulted in a significant increase of dried root biomass, with a high plumbagin production (1.09 mg/g DW) that was 1.96-fold higher than the level in the control. Based on biomass of one cultured flask, the calculated amounts of plumbagin produced by the 5- and 10-day old treated roots were 0.59 and 0.37 mg/250 mL-flask, respectively, which were 4.2 and 2.6 fold higher from the level in the control (0.14 mg/250 mL-flask) (Figure 4.8). Treatment at 10-day old resulted in increased extracellular secretion of plumbagin. This may affect the growth of the root cultures due to the toxicity of plumbagin. The extracellular release of plumbagin along with gamma ray irradiation might also damage the cell structure causing browning effects and cell death. While, the 5-day old root cultures showed healthy

growth and stable roots for further continuous subculture, with high plumbagin content.

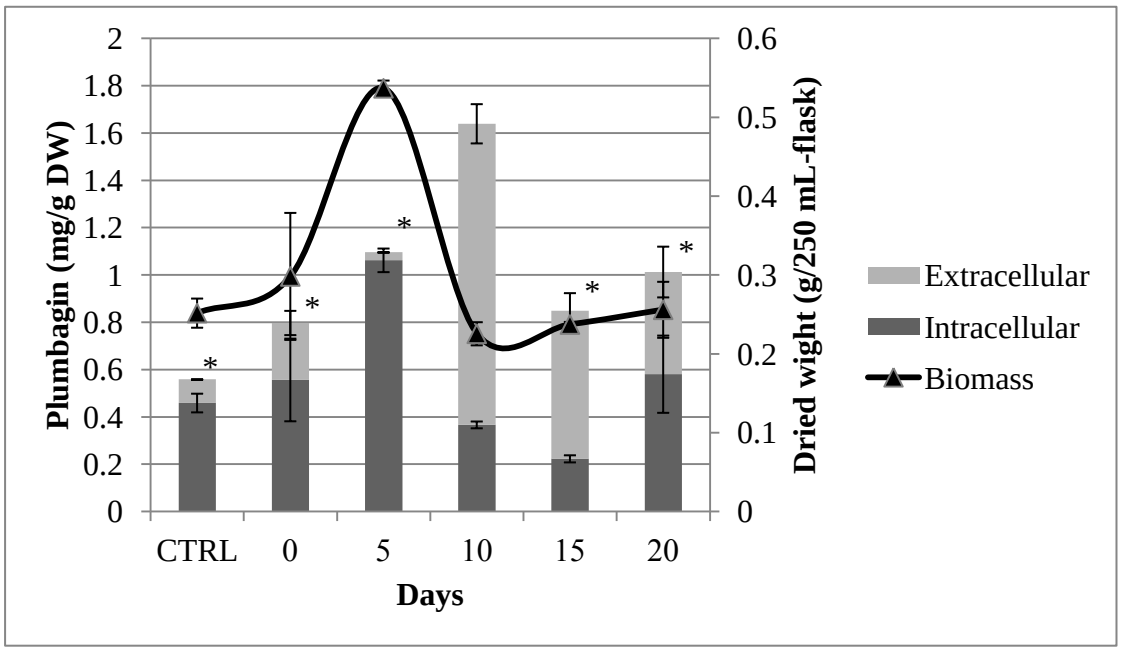


Figure 4.7 Effect of 20-Gy irradiation on biomass and plumbagin production *in P. indica* root cultures treated on different days. CTRL is untreated group. Data are mean ± SE of n = 3**.** *significant difference (P<0.05) when compared with the 10-days groups.

Ten day old root culture showed highest content in extracellular with decreased in cell biomass. In this case, a two-phase culture or *in-situ* extraction may be used for further studies to improve the cell viability and plumbagin production of *P. indica* root cultures. The increase of the plumbagin content in the cultured media leads to the low viability of the root cultures, possibly due to the effects of plumbagin that is released into media. Such effects and cell growth retardation have been reported in *Impatiens balsamina* culture treated with methyl jasmonate (Sakunphueak & Panichayupakaranant, 2010). It has been suggested that browning coloration might also be due to other phenolic compounds as a response to elicitor-induced stress (Zhao *et al*., 2001). Furthermore, it has been reported that *in situ* extraction and elicitation

cell treatment had a synergistic effect on the stimulation of plumbagin production in suspension culture of *P. rosea* (Komaraiah *et al*., 2003).

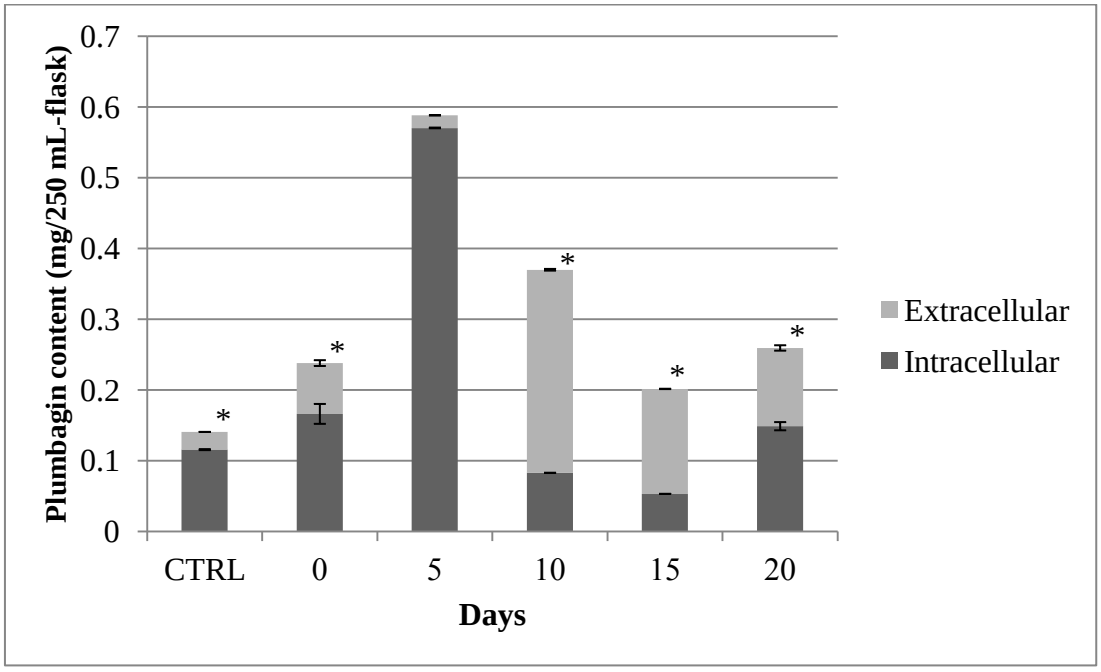


Figure 4.8 Effect of 20-Gy irradiation on plumbagin content in one root cultured flask. CTRL is untreated group. Data are mean ± SE of n = 3**.** *significant difference (P<0.05) when compared with the 5-days groups.

6. Study on the naphthoquinone production profile in *P. indica* root cultures

The naturally grown *P. indica* roots was found rich with content of the naphthoquinone; plumbagin (**1**) then the other two naphthoquinones, elliptinone (**2**) and 3, 3'-biplumbagin (**3**). HPLC analysis of the extracts from both untreated and treated root cultures showed that plumbagin was the major naphthoquinone (Figure 4.9), while the other two naphthoquinones were not detected.

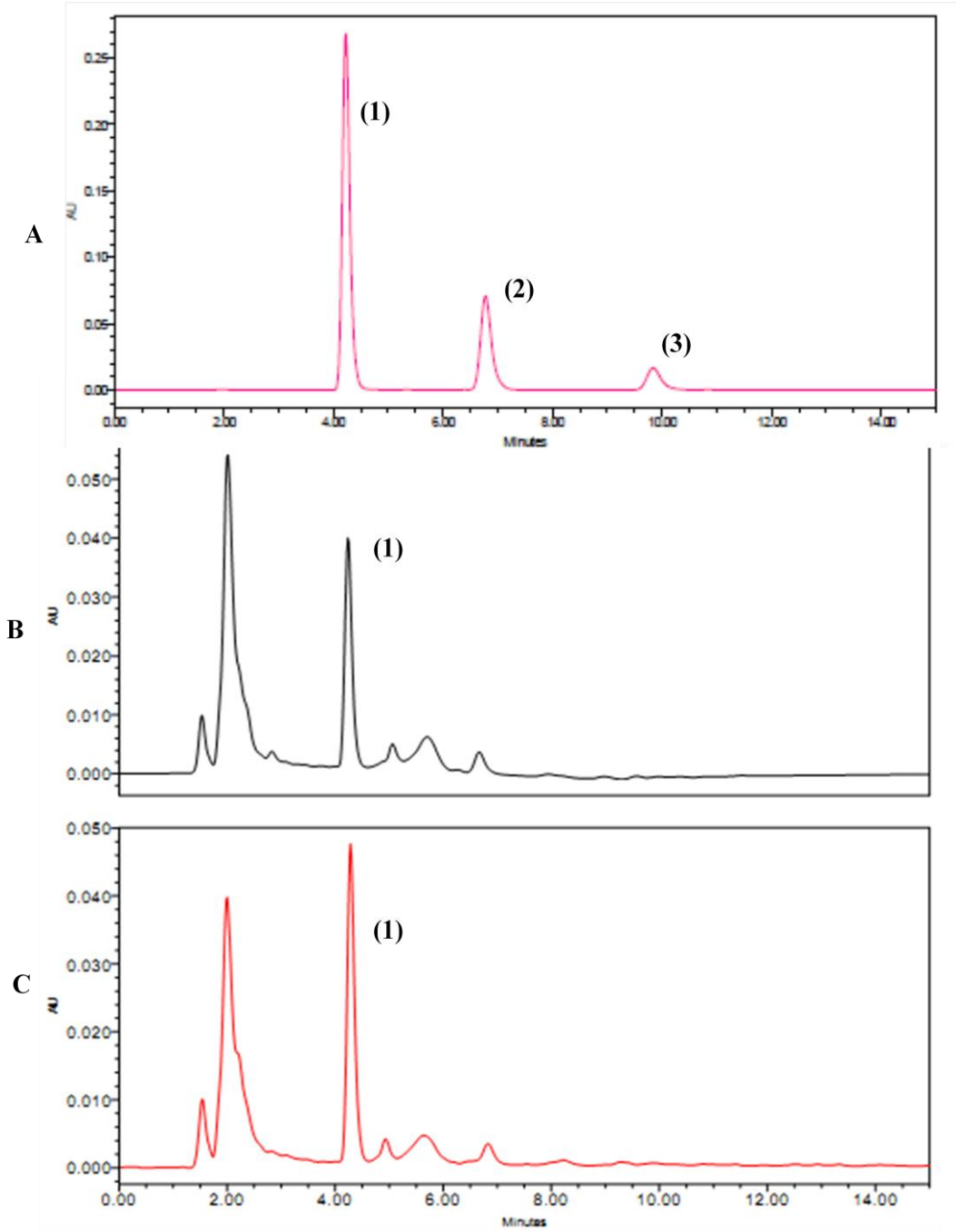
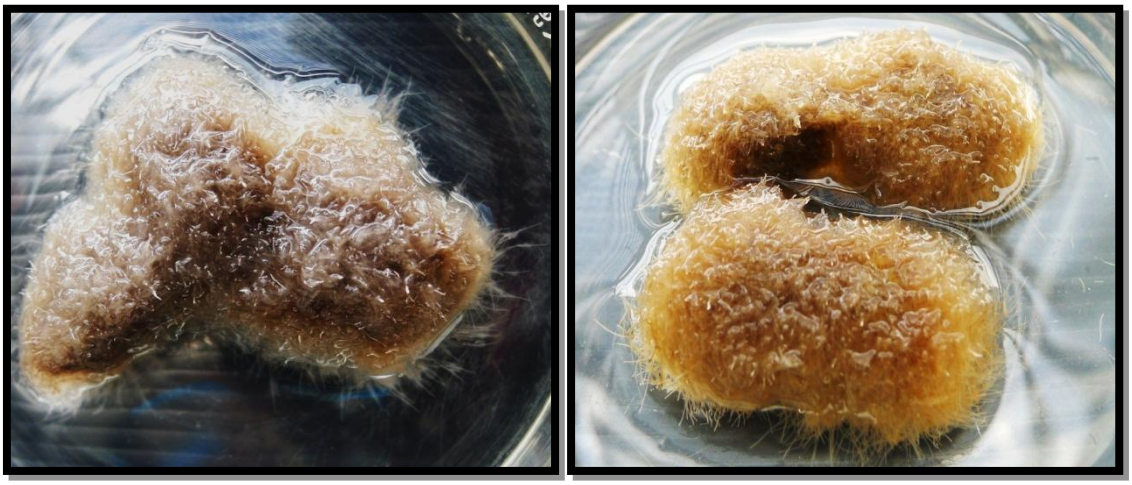


Figure 4.9 HPLC chromatograms of (A) the standard naphthoquinones and the extracts from (B) untreated and (C) 10-Gy treated *P. indica* root cultures

7. SEM analysis of *P. indica* root cultures

Gamma ray is a form of ionizing radiation that interacts with atoms and molecules to produce free radicals. These processes can damage or excite various important compounds. Low or high doses of ionizing radiation were used to stimulate or inhibit seed germination, plant growth and productivity in various plants, respectively (Shamsi and Sofajy, 1980; Kim *et al.*, 2005; Wi *et al.*, 2005; Wi *et al.*, 2007; Hasbullah *et al.*, 2012). However, the mechanisms for these responses are not well known and are limited. By determining the histological changes to root cells at morphological level, we can gain insight into the mode for ionizing radiation. Therefore, our objectives are to study the effects of low dose (20 Gy) of gamma ray irradiation on the cells of *P. indica* root cultures. According to a morphological observation of the root cultures found that only the colors of the treated and untreated root cultures were different (Figure 4.10).



A **B**

Figure 4. 10 The untreated (A) and 20 Gy-treated (B) *P. indica* root cultures

Furthermore, with such a change in its morphological view, we then observed its histological surface view. The electron micrograph of control root surface was clear and with smooth differentiation of epidermal cells (Figure 4.11A). Whereas, a radical expansion of root epidermal cells in the elongation zone as well as some

burst in epidermal cells with occurrence of root hairs or trichomes were observed in the 20 Gy elicited roots at the same magnification (Figure 4.11B). Such characteristic change in the histological might be cause of gamma ray irradiation and radial expansion of epidermal cells which initiates outgrowth of roots hairs (Nagata *et al.*, 2004).

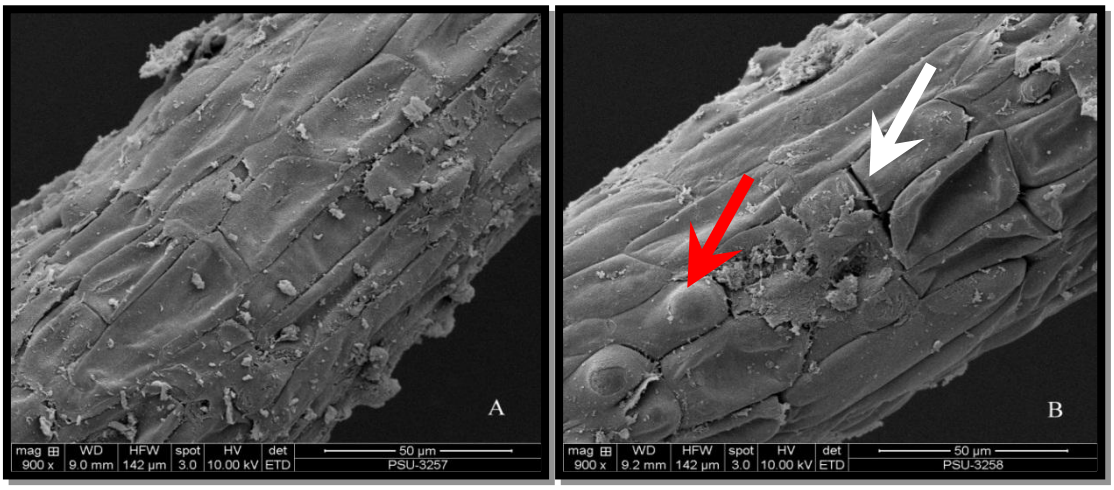
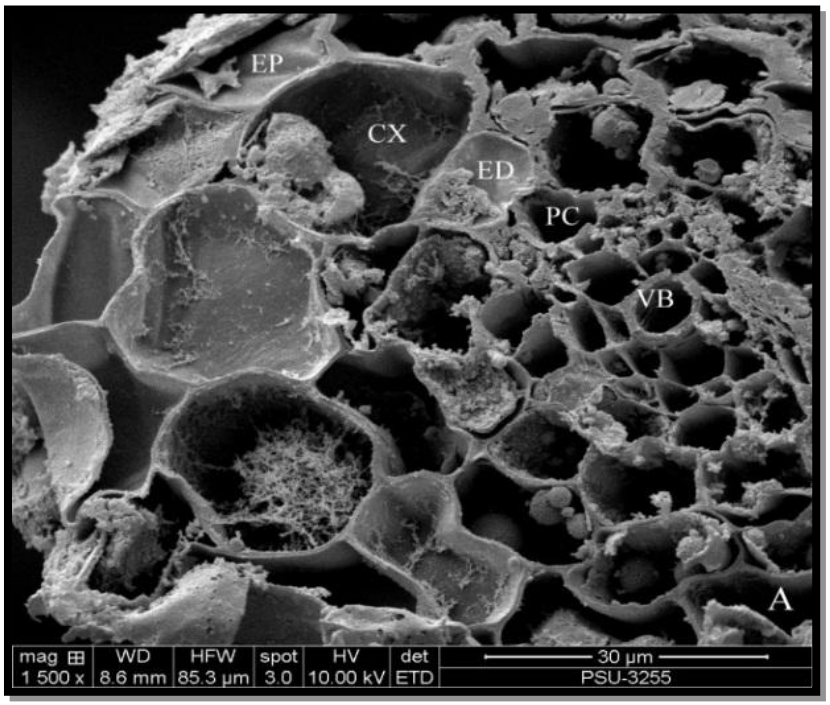
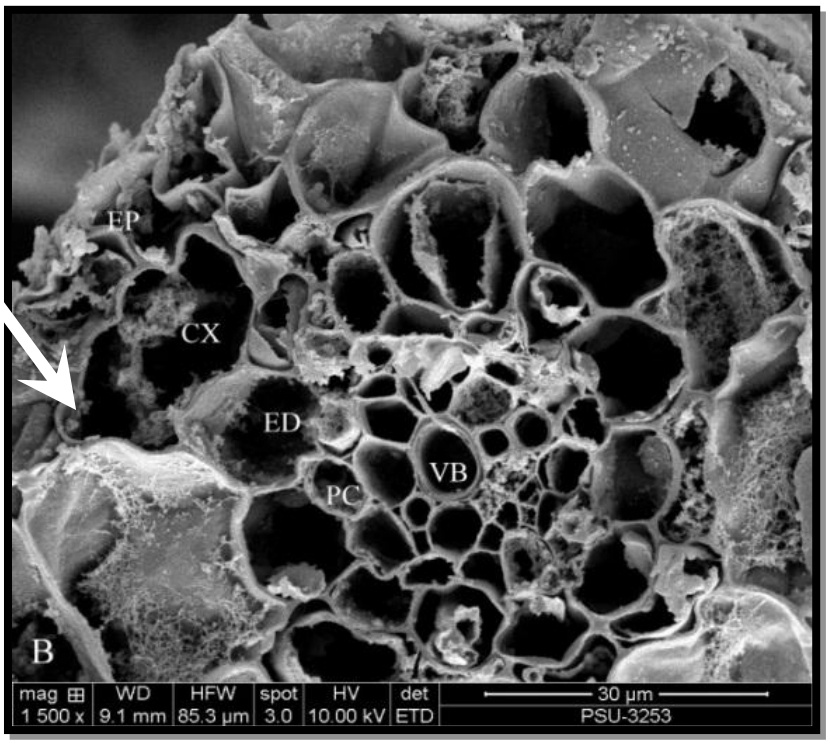


Figure 4. 11 Surface histology of (A) the untreated and (B) 20 Gy-treated *P. indica* root cultures. Bar = 50 µm. A red arrow indicates a root hair or trichome. A white arrow indicates a burst of epidermal cells.

The histological studies of the cultured roots by SEM indicated that an ultrastructure of the cultured roots consisted of single layers of epidermis, cortex, endodermis and pericycle surrounding a vascular bundle (Figure 4.12A). *Arabidopsis* roots were found with similar observation (Nagata *et al.*, 2004; Wi *et al.*, 2005). The epidermal and cortical cell layers of the 20 Gy-treated roots were disturbed or wide open like bursts and expanded radically (Figure 4.12B). Such changes might be due to gamma ray irradiation effects.



A



B

Figure 4.12 SEM micrograph of (A) untreated and (B) 20-Gy treated *P. indica* root cultures. Bar = 30 µm; EP-Epidermis; CX-Cortex; ED-Endodermis; PC-Pericycle and VB-Vascular bundles.

Therefore, we conclude that histological appearance of *P. indica* roots consist of organized cell layers i.e. epidermis, cortex, endodermis, pericycle and vascular bundles. The gamma radiation due to its radiolysis effect and radial expansion of epidermal cells might be possible for causing the occurrence of root hairs or trichomes**.** Similar properties were observed in with *Arabidopsis* roots exposed to high dose of gamma radiation (Nagata *et al.*, 2004; Wi *et al.*, 2005).

It has been reported that that ethylene production can be induced by gamma radiation of plants. This phytohormone, ethylene is said to plays a key role in the radial swelling in *Arabidopsis* roots (Nagata *et al.*, 2004). Ethylene regulates root elongation, as well as root hairs initiation and expansion. The morphological changes like expansion and swelling of *P. indica* roots might be cause of the ethylene production induced by gamma ray irradiation. Cell expansion and root hair differentiation are controlled by active oxygen species (AOS) (Foreman *et al.*, 2003). The generation of a large amount of AOS may induce many physiological changes in root tissues (Nagata *et al.*, 2004). Therefore, we presumed the action of ethylene production participates in gamma ray irradiation induced root morphological changes and is mediated by AOS. However, further studies are needed to prove the above mentioned.

8. Effects of subculture on the stability of *P. indica* root cultures

The main drawback of cultured plant cells is lower yields, stability of the cell lines, inconsistency in the production and the storage of the metabolites within the cells or vacuoles (Komaraiah *et al*., 2003). The 20-Gy treated root cultures were therefore subcultured to observe the stability of growth and plumbagin production in the three generation batch. It was found that subculturing of the root cultures until the third generation showed an increase in plumbagin production without any effect to their growth (Figure 14.13). However the root growth showed variation in each cycle of subcultures. This may be due to the extracellular secretion of plumbagin, as a result causing cell retardation and decline in production as found in *Impatiens balsamina* root cultures (Sakunphueak & Panichayupakaranant, 2010). These results showed that the 10-Gy treated root cultures were stable at least three cycles of subcultures.

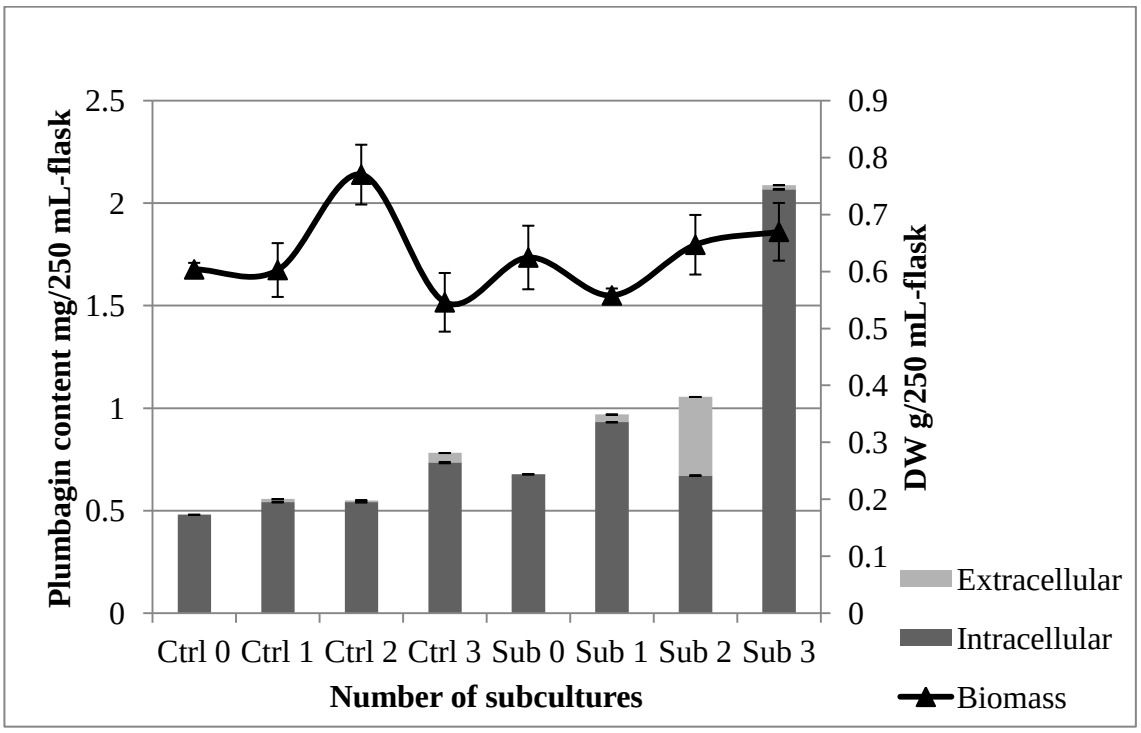


Figure 4.13 Plumbagin production and biomass of *P. indica* root cultures during three cycles of subculture. Data are mean ± SE of n = 3. Ctrl 0 = Initial control root cultures. Ctrl 1, 2 and 3 = first, second and third subculture of the untreated root cultures. Sub 0 = Initial elicited root cultures. Sub 1, 2 and 3 = first, second and third subculture of the 10-Gy treated root cultures.

CHAPTER 5

CONCLUSIONS

From this research work the following conclusions can be drawn:

1. Root cultures of *P. indica* were established from young leaf explants of *P. indica* and maintained in liquid B5 medium supplemented with 0.1 mg/L NAA. A growth cycle of the root cultures spent 24 days. The root cultures reached to a stationary phase at day 20, and gave the highest dry biomass (0.39 g/250-mL-flask), which was 3 times the biomass of the inoculated roots. Plumbagin was an only naphthoquinone detected in the root cultures. Plumbagin was initially accumulated in a linear phase (after day 12) and actively biosynthesized until reaching the stationary phase. The highest content of plumbagin was observed at day 20 (0.86 mg/250-mL-flask).

2. Effect of low dose of gamma ray irradiation (0, 5, 10, 15, 20, and 25 Gy) as well as age of the root cultures for elicitation (0, 5, 10, 15, and 20 days) on plumbagin production of *P. indica* root cultures was determined. Although all treated doses showed positive effects on plumbagin production, the optimum dose of gamma ray for increased plumbagin production by *P. indica* root cultures was 20 Gy (1.04 mg/g DW).

3. The appropriate age of root cultures for maximum production of plumbagin (1.64 mg/g DW) was found to be 10 days, which was 2.94-fold higher than the level in the control (0.56 mg/g DW). However, treatment of 5-day old root cultures resulted in a significant increase of dried root biomass, with a high plumbagin production (1.09 mg/g DW) that was 1.96-fold higher than the level in the control. Based on biomass of one cultured flask, the calculated amounts of plumbagin produced by the 5- and 10-day old treated roots were 0.59 and 0.37 mg/250-mL flask

DW, respectively, which were 4.2 and 2.6 fold higher from the level in the control (0.14 mg/250-mL flask DW).

3. *P. indica* root sections were studied by SEM, focusing on the histological studies in the roots cultures exposed to gamma dose of 20 Gy. The histological anatomy of *P. indica* consists of single layers of epidermis, cortex, endodermis and pericycle surrounding a vascular bundle. The 20 Gy treated root showed, the radical expansion of root epidermal cells in the elongation zone of the roots and some burst in epidermal cells. Such effect of gamma ray irradiation causing radial expansion of epidermal cells initiating outgrowth of roots hairs or trichomes was observed.

5. A high degree of stability for plumbagin production by the elicitated root cultures was achieved during three cycles of subculture.

6. The established *P. indica* root cultures and the elicited root cultures showed only plumbagin as the naphthoquinone profiles.

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List of Publication and Proceeding

List of Publication

Jaisi, A. Sakunphueak, A and Panichayupakaranant, P. 2012. Production of plumbagin by *Plumbago indica* root cultures. *Instasci Journal of Pharmaceutical* Sciences Research (Submitted).

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List of Proceeding

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