PRODUCTION OF PLUMBAGIN BY HAIRY ROOT, CALLUS AND CELL SUSPENSION CULTURES OF *Plumbago indica* L.

INTRODUCTION

Medicinal plants are gaining great interest in pharmaceutical industries for the production of high valued secondary compounds (Rout *et al*., 2000; Das and Rout, 2002). *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 pharmacological properties such as anticancer (Parimala and Sachdanandam, 1993), antimicrobial (Didry *et al*., 1994), antifertility (Bhargava, 1984) and insecticidal properties (Kubo *et al*., 1983).

The plumbagin detection in various parts of *P. indica* L. is carried out by high performance liquid chromatography (HPLC) which is accurate and sensitive (Stensen and Jensen, 1994), but expensive, time consuming and inconvenient for routine assay (Dankwardt and Hock, 2001). Thus, immunological methods have become increasingly popular as the screening tools for the determination of the particular compound which can be surveyed and characterized according to the selectivity of an antigen-antibody affinity. Immunological methods could provide simple, inexpensive and fast way for measuring a number of samples if suitable antibodies are available (Beale, 1999; Chan and Ho, 2002). There are several reports of immunolocalization of secondary metabolites in plant cell and tissue (Schraut *et al*., 2004; Brisson *et al*., 1992; Santiago *et al*., 2000) mainly for the study of proteins or enzymes involved in biosynthetic pathways of secondary metabolites (Saslowsky and Shirley, 2001; Canto-Canché *et al*., 2005). However, very little is known about the site of plumbagin accumulation at the tissue, cellular and subcellular levels. Thus, in order to understand the pattern of plumbagin accumulation in plant tissue, the polyclonal antibodies were raised against plumbagin–BSA conjugate and used for the immunolocalization of plumbagin.

The therapeutic use of plumbagin is limited due to its insufficient supply from the natural sources as the plants grow slowly and take several years to produce quality roots (Kitanov 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 compounds.

Many of the previous experiments have reported the accumulation of plumbagin in Droceraceae family such as *Drosophhyllum lusitanicum* L. (Nahálka *et al*.1996a and b; Nahálka *et al*., 1998), *Drosera natalensis, D. capensis* L. (Crouch *et al*., 1990) and *D. gigantean* (Budzianowski, 2000). Regard to the family Plumbaginaceae, there are some reports for the production of plumbagin from callus and cell suspension cultures of *Plumbago indica* L. (Komaraiah *et al*., 2001; Komaraiah *et al*., 2002). However, there are still several problems in the production of this phytochemical compound by cell cultures such as the instability and slow growth of the cell lines and low yields of plumbagin (Choosakul, 2000). 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 (Roa and Ravishankar, 2002). These strategies are interesting for enhancement of the plumbagin production from cell and tissue cultures of *P. indica* L. which may leads to an opportunity for large scale production.

The main objectives of this study were;

1) to develop the immunolocalization technique for plumbagin detection in *P. indica* L.
2) to investigate the plumbagin production from *P. indica* L. hairy root, callus and cell suspension cultures
3) to enhance plumbagin production using biotic and abiotic elicitors.

LITERATURE REVIEW

***Plumbago indica* L.**

1. **Botanical Aspects of *P. indica* L.**

The genus *Plumbago* consists of about 20 species of herbs native to the tropical regions of the world. *P. indica* L. (Figure 1) or Chettamuun Phloeng Daeng (Thai name)**,** a native of South Asia, is a small shrub. This plant grows to a height of up to 0.8-1.5 m. Leaves are alternate, oval-shaped, hairless, mid to deep green, and up to 3-5 cm wide, 6-10 cm long. Stem is tubular with red nodes and has got red flowers, corolla red, its segments manifestly apiculate; calyx 8-9 mm long, glabrous, gland-bearing throughout its length; ovary ovoid-oblong; style-base short-hairy; fruit unknown; spikes never corymbose, rather lax, their rachis 10-30 cm, glabrous (Saralamp *et al*., 1996; van Steenis, 1949).

P. indica L. can be propagated both by sexual and asexual reproduction. However, as it produces very small amount of seed and its seed is poor germination. The asexual propagation, both root and stem cutting, is the most commonly used (Krishnan, 2003).

2. **The Use of *P. indica* L.**

Roots of *P. indica* L. are used as raw material for extracting plumbagin in China and other countries (Grieve, 1995). The root is acrid, vesicant, abortifacient and stimulant. Applied in bland 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 (Watson and Dallwitz, 1992)**.**

3. Chemical Constituents of *P. indica* L.

As reported by Farnsworth (1999), several groups of phytochemical have been found in different plant parts of *P. indica* L. The quinoid comprising plumbagin was identified in roots, rootbark and aerial parts of this plant. Other chemical constituents found in this species together with plumbagin have been presented in Table 1.

Table 1 Chemical constituents of *P. indica* L.

Plant parts	Chemical group	Chemical substance
Root	benzenoid	benzene
	carbohydrate	fructose, glucose
	flavonoid	leucodelphidinin
	quinoid	naphthoquinone,1-4
		naphthoquinone, alpha
		plumbagin
	alkaloid	naphthylamine
rootbark	alkane	arechidyl alcohol
	carbohydrate	glucose
	lipid	lignoceric acid, linoleic acid
		oleic acid
	quinoid	plumbagin
	steroid	sitosterol, beta
aerial parts	steroid	campesterol
		sitosterol, beta
		stigmasterol
	quinoid	plumbagin
		6-hydroxyplumbagin
	flavonoid	plumbagin

Source: Farnsworth (1999)

Figure 1 *Plumbago indica* L. (Plumbaginaceae)

Source: van Steenis (1949)

Plumbagin

1. Structure and Chemical Properties

Plumbagin, 2-methoxy-5-hydroxy-1, 4-naphthoquinone ($C_{11}H_8O_3$), is a natural product found in roots of *P. indica* L. with a molecular weight of 188.18. Plumbagin occurs as yellow pigment and its melting point is 78-79 ^{0}C. It is slightly soluble in hot water and well soluble in alcohol, acetone, chloroform, benzene and acetic acid. It is highly toxic and corrosive (Sigma-Aldrich, 1999). The structure of plumbagin is shown in Figure 2.

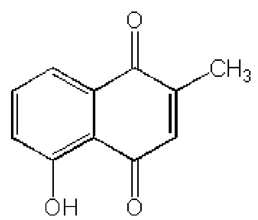

Figure 2 The chemical structure of plumbagin
Source: Sigma-Aldrich (1999)

Concerning the physio-chemical properties, plumbagin shows its ultraviolet spectrum with absorption bands at 212, 266, 410 and 423 nm (log ε 4.35, 3.92, 3.39, and 3.40) and for nuclear magnetic resonance spectroscopy (NMR), plumbagin shows δ at 184.2 (s, c1), 149.2 (s, c2), 135.3 (d, c3), 189.8 (s, c4), 160.7 (s, c5), 123.8 (s, c6) 135.3 (d, c7), 188.8 (d, c8), 131.7 (s, c9), 114.7 (s, c10), 16.2 (s, c11) (Nahálka *et al*., 1996b).

2. Natural Occurance of Plumbagin

Plumbagin occurs in several plant species of the family Plumbaginceae and Droseraceae. 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 (Botanical Dermatology Database, 1999 a, b).

3. 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).

4. Biological Activity of Plumbagin

Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a natural naphthoquinone showing a broad range of pharmaceutical activities. It has been reported to antifertility by stimulating the uterus to constrict and lead to abortion. Giving a dose of 10 mg/kg ip for 60 days has been shown to cause selective testicular lesions in dogs. The wet weights of testis and epididymides were decreased. (Bhargava, 1984). Plumbagin administered by incubation to albino female rat at 10 mg/kg for 15 days significantly inhibited mating and prolonged duration of estrus cycle and dieatrus phase (Premakumari *et al*., 1977). Plumbagin has also found to have anticancer by inhibition of tumor cell growth (Parimala and Sachdanandam, 1993). Plumbagin exhibited antimicrobial activity. The growth of *Staphyloccocus aureus* and *Candida albicans* was completely inhibited. It was effective against *Salmonella gallinarum, S. typhimurium, Escherichia coli*, *Proteus vugaris* and *Bacillus subtilis* (Desta, 1993; Didry *et al*., 1994; Ahmad *et al*., 1999; Paiva *et.al*., 2003). It also delayed the germination of *Aspegillus flavus* and were capable of completely inhibiting growth at high concentration (50 ppm) (Mahoney *et al*., 2000). Plumbagin has many effects against insects such as insect feeding deterrent, reduced the growth rate and the ability of mating (Satyanarayana and Gujar, 1999; Villavicencio and Perez-Escandon, 1992). It has been shown to inhibit insect development, mainly through interfering with hormonal processes of moulting and effective chitin-synthetase inhibitor (Kubo *et al*., 1983). Therefore, it can be utilized for controlling insect pests in agriculture (Nahálka *et al*., 1996a).

5. 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). While most naphthoquinones are derived from Shikimate pathways, plumbagin from *Plumbago* spp. is derived from six C_2 units (Durand and Zenk, 1971). The biosynthetic pathway of plumbagin is presented in Figure 3.

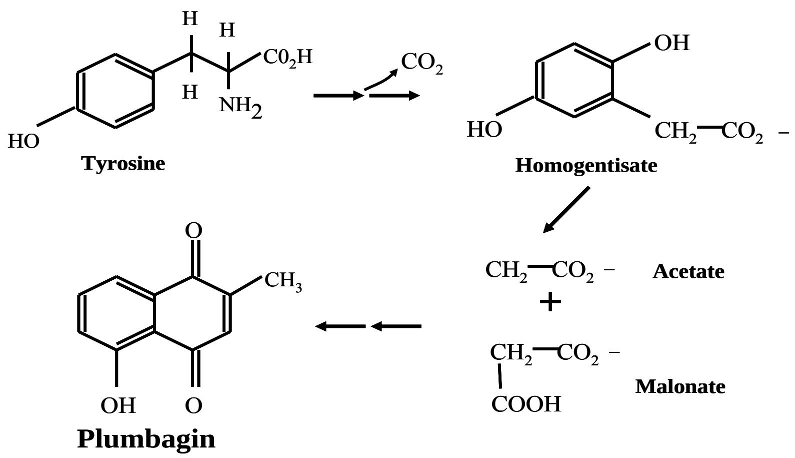

Figure 3 The biosynthetic pathway of plumbagin
Source: Durand and Zenk (1971)

The Application of Immunological Methods in Plant Secondary Metabolite Studies

The main biological function of secondary metabolites may be as chemical defense agents. They are directed at a variety of microorganisms (virus, bacteria and fungi), other plants (allelopathy) and phytophagous animals (insects and vertebrates) (Swain, 1997). Plumbagin is considered to be a secondary metabolite. However its function in the intact plant remains to be defined and very little is known about the site of plumbagin synthesis and storage. *Plumbago* spp. produced plumbagin which is highly toxic (Singh and Udupa (1997).

The cytotoxicity of plumbagin may also indicate a role in plant defense, however, this is merely hypothetical. Many secondary metabolites are known for their cytotoxicity and therefore, their synthesis must be coordinated with other cellular functions and their accumulation restricted to particular cellular compartments (Renaudin, 1989). When a metabolite is found in a particular plant part, it does not necessary mean that it has also been synthesized there. Three possibilities may be distinguished: i) a compound is stored in the same cell in which it had been formed, ii) a metabolite is accumulated in cells adjacent to the cells in which it is synthesized, and iii) a product is stored in specialized organs isolated from the plant part of it original synthesis (Wink, 1987).

For more information about there site of plant secondary metabolites synthesis and storage, immunological methods have become increasingly popular as screening tools for plant secondary metabolites by applying immunolabeling to microscopic sections of plant tissues. Various kinds of plant secondary metabolites have been localized *in situ*, and their formation was examined (Krell, 1993; Nakashima *et al*., 1997). The basis of all immunological techniques is the availability of the antibodies with high specificity for the substance of interest. In order to use the immunological techniques to study plant secondary metabolite, it is necessary to have antibody specific to certain substance (Beale, 1999). However, the specific antibody can be achieved by several immunological techniques.

Immunofluorescence is a method for the detection and localization of specific antigens in sera or in tissue structures visible by ordinary light microscopy which can be easily accomplished using antibodies labeled with fluorescent dye. When irradiated with short wavelengths (ultraviolet, blue, or green excitation) of light, fluorescent substances emit electromagnetic radiation in the visible region. This property is known as primary or autofluorescence. The objects such as tissue background will emit no fluoresence. Light emission from a fluorochrome is called secondary fluorescence (Clausen and Green, 1997).

Application of fluorescent antibody immunohistochemistry allows the detection and localization of plant secondary metabolites easy to be visualized. The majority of plant secondary metabolites are low molecular weight haptens. Then, several aspects of immunocytochemistry for plant secondary metabolites need to be considered: i) method for antibody detection, ii) tissue preparation to preserve both cellular fine structure and antigenic activity, and permit access of labeled antibodies to cellular sites; iii) kinds of marker or probe conjugated to the antibody that are suitable for detection in plant tissues by light or electron microscopy (Knox, 1982).

1. Preparation of Antigen and Production of Antibody

For the development of polyclonal antibodies, it is recommended to use very pure antigen due to its high specificity to antibody. In general, some antigens are immunogenic; that is, they will elicit an immune response in a tested animal. However, some small antigens (MW< 3000 Daltons), so called haptens, are not immunogenic. To improve the immunogenicity of haptens, it is possible to couple them with an immunogenic carrier, usually proteins such as bovine serum albumin (BSA), keyhole limpet hemacyanin (KLH) (Harlow and Lane, 1988). Three methods are generally used for coupling reaction between antigen and carrier: i) glutaraldehyde coupling *via* amine group ii) the mixed anhydried reaction to specifically activate carboxyl groups before reacting with amine groups; iii) the carbodiimide reaction to couple carboxyl groups to form intermediates which are then capable of reacting with amino group (Edwards, 1996). The desired properties of the antibodies can be successfully by modification of various factors, e.g. nature of immunogen (protein or hapten), immunogen dose, immunization period, method of application, adjuvant and modulation of immune system (Harlow and Lane, 1988).

The naphthoquinone groups of plant secondary metablite act as hapten (Hatton, 1995). These low molecular weight chemicals are not in themselves immunogenic, but after binding with carrier proteins, hapten-protein conjugates emerged, which are capable of inducing specific immune response (Beale, 1999). Production of polyclonal antibody for the detection of protein modification by

1, 2 naphthoquinone in animal tissues has been established by Zheng and Hammock (1996). The competitive enzyme-linked immuno sorbent assay (ELISA) showed that the produced antibodies specifically recognized 1-2 naphthoquinone. Plumbagin is also a low molecular weight hapten and very toxic (Sigma-Aldrich, 1999), hence, it has to be coupled with the carrier protein to get the good immunogenicity. To date, there has not been any study on the production of the antibody specific for plumbagin.

2. Immunolocalization of Plant Secondary Metabolites

During the 1970s, immunohistochemical methods for localization of macromolecules such as enzymes, storage proteins and polysaccharides became established in plant science (Beale, 1999). They involved coupling and labeling the antibody to the component molecules of interest within a tissue section. The antibody may be polyclonal or monoclonal antibody. The coupling site within the tissue is revealed by tagging antibody with a fluorescent dye or enzyme for studies using light microscopy or with colloidal gold for electron microscopic work for plant antigens (Bergman *et al.*, 1996). The use of these methods to localize phytochemical substances are made difficult by the need of preventing these small molecules from diffusing away from their *in vivo* subcellular locations, or even being lost by dissolution in solvents, especially during sample preparation (Beale, 1999). Nevertheless, there have been a number of reports of the successful immunolocalization of chemical substances in plant cells after appropriate tissue preparation. In the case of hydrophilic compounds, diffusion may be overcome by rapid freezing and freeze substitution (Fisher, 1972; Zavala and Brandon, 1983) or by freeze drying (Luckner *et al.*, 1980), cryotechniques (Russin *et al.*, 1995). In plant tissues, antigen-antibody reaction can be disturbed by lectins since some lectins may bind IgG (Krell, 1993). For example, Nahálková *et al.* (2001) used immunological techniques such as immunofluorescence and immunolabeling for detection of lectin binding on the fungal cell wall during infection in all parts of pine seedling. Immunolocalization is also a well developed method used to localize secondary metabolites within plant tissue such as taxol in the cell wall of bark, wood and leave of *Taxus cuspidate* (Russin *et al.*, 1995); ascorbic acid and glutathione in roots of

Cucurbita maxima (Liso *et al*., 2004); abscisic acid in maize roots (Schraut *et al*., 2004); flavonoid enzyme in the epidermal and cortex cells of the elongation zone and the root tip of *Arabidopsis* and plant cells (Brisson *et al*., 1992; Santiago et al., 2000) mainly for proteins or enzymes involved biosynthetic pathways of secondary metabolites (Saslowsky and Shirley, 2001; Canto-Canché *et al*., 2005). However, the production of antibody for localization of plumbagin in *P. indica* L. has not been reported.

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.

1. Nutrient and Environmental Factors Influencing the Accumulation of Secondary Metabolites

1.1 Manipulation of Culture Media

The expression of many phytochemical compounds is easily affected by external factors, such as nutrient levels, stress factors and plant growth regulators. Over the last decade, several approaches involving nutrient and environmental condition optimizations have been studied to enhance the production of secondary metabolites in plant cell and tissue cultures. Manipulation of culture media is an important area given attention for increasing secondary metabolite accumulation. (Rao and Ravishankar, 2002). Dong and Zhong (2002) reported that taxine accumulation in *Taxus chinensis* could be done by sucrose feeding. In callus cultures of *Populus* sp. and *Daucus carota*, the replacement of 2,4-D by NAA or indole acetic acid (IAA) had been shown to enhance the production of anthocyanins (Rajendran *et al*., 1992).

The application of these strategies to callus and cell culture of *P. indica* L. led to an enhancement in plumbagin production as well (Komaraiah *et al.,* 2003). However, the increase in production is at most 3-4 times of the basal level which is not enough to be able to produce this chemical commercially.

1.2 Optimization of the Culture Conditions

Environmental factors as well as nutrient composition of culture media exert control on the *in vitro* production of secondary metabolites. Furthermore, the effects of light, temperature and pH of the culture medium have also been studied as a mean to enhance secondary metabolite production. Light, temperature and pH of the medium are important parameters in culture incubation. Light is essential for morphogenetic process such as shoots and roots initiation and somatic embryogenesis. Both quality and intensity of light as well as the photoperiod are very critical to the success of certain culture experiments. The temperature usually employed in the culture incubation room is 25 ^{0}C (Murashige, 1977). The pH of medium is usually adjusted between 5 and 6 before autoclaving. The pH of the medium decreases during ammonia assimilation and it increases during nitrate uptake (McDonald and Jackman, 1989). The pH changes in cell culture caused by metabolic reactions are necessary for nutrient absorption and maintenance of culture growth (Figueiredo *et al*., 2000).

Light plays an important role in the growth, differentiation, tissue organization and production certain primary and secondary metabolites. Light was observed to have a marked influence in the growth and accumulation of secondary metabolites.

For example, the thiophene in hairy roots of *Tagetes patula* where biomass yield decreased in the presence of light and the composition of thiophene was altered significantly (Mukandan and Hjortso, 1991). The *Ambrosia artemisiifolia* hairy root cultured at light condition showed a decrease in biomass yield and complete cessation of thiarubrine A (TA) accumulation. Light has the effect of switching the metabolic pathways on the formation of this secondary metabolites by changing in its color, from light pink when hairy roots were grown in darkness to red pigment when culture grown under light (Bhagwath, 1997). The biosynthesis of shikonin in *Lithospermum erythrorhizon* is strongly inhibited by light, eventhrough other environmental conditions are optimized (Yazaki *et al*., 2001). Contrary to light-inactivated secondary metabolism, there are several plants stimulated by light such as isoflavones from callus culture of *Genista* species (Luczkiewicz and Głód, 2003).

There have been reported the effect of a lower pH medium on the production and release of secondary metabolites. Lowering the pH to 3.5 and 4.5 reduced the accumulation of alkaloid in the hairy roots of *Brugmansia candida*, but at a pH of 4.5, the release increased significantly. Acetic acid and citric acid stimulated the release of scopolamine and hyoscyamine (Pitta-Alvarez and Giulietti, 1999).

2. Elicitor Treatment as Strategy to Improve Production of Secondary Metabolites

Elicitors are chemicals from various sources that can induce physiological changes of the target living organism. 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).

Elicitors can be categorized, based on their origin, into two major groups; biotic and abiotic. The biotic elicitors range from macromolecules such as oligosaccharides (e.g., chitin, chitosan) polysaccharides derived from plant cell wall (e.g. pectin or cellulose), phospholipids and glycoproteins, to small molecules such as hydrogen peroxide, ethylene, methyl jasmonate, and salicylic acid. Abiotic elicitors usually refer to inorganic salts such as mercuric chloride ($HgCl_2$), copper sulfate ($CuSO_4$), calcium chloride ($CaCl_2$), and vanadyl sulfate (VSO_4) including mechanical stress agents such as ultraviolet radiation, wounding and chemicals that disturb membrane integrity. These elicitors interact with plant cells through different and complex mechanisms (Darvill and Albersheim, 1984; Benhamou, 1996).

2.1 Mechanism of Elicitation in Plant Cells

Several researches have focused mainly on the biotic elicitors, carbohydrate elicitors, 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 its 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).

Chitin and chitosan are effective elicitor that are extensively used. Chitosan treatment greatly increased the membrane permeability of suspension cultured cells (Brodelius *et al*., 1989). Production of phytoalexins and the generation of hydrogen peroxide are also responses of plant elicited with chitosan. There is evidence on the signal transduction pathways involved in the elicitor actions. It has been reported that chitosan stimulates the accumulation of jasmonic acid, a signal molecule related to defense-gene regulation. Morever, treatment cultures of *Rubia tinctorum* enhanced the anthraquinone production (Vasconsuelo *et al*., 2004). The mechanism of chitosan stimulation the anthraquinone which is a secondary metabolite was shown in Figure 4. The signalling mechanism by chitin or chitosan increase the plumbagin production in *P. indica* L. is unknown.

The transduction of elicitor signals in plant cells may a usefull mechanism in secondary metabolite production. The secondary messengers are generated and led to the activation of protein kinase cascades which may activate the biosynthetic ability for specific plant products. A mechanism for biotic elicitation in plants may be summarized on the basis of elicitor-receptor interaction. When plant or plant cell culture is challenged by the elicitor a rapid array of biochemical responses occur inseveral steps described below (Radman *et al*., 2003).

- Binding the elicitor to the plasma membrane receptor
- Changes in ion flux across the membrane: Ca^{2-} influx to the cytoplasm from the extracellular environment and intracellular Ca^{2-} reservoirs; stimulatiom of K^- and Cl^- efflux
- Rapid changes in protein phosphorylation patterns, protein kinase activation, mitogen activated protein kinase (MAPK) stimulation, G-protein activation
- Synthesis of secondary messengers, Ins P_3 and diacyl-glyceral (DAG), in which, mediating the intracellular Ca^{2-} release and nitric oxide and octadecanoid signaling pathway
- Cytoplasm acidification caused by H^+-ATPase inactivation, decrease in membrane polarization and extracellular pH increase
- Activation of NADPH oxidase responseible for ROS and cytosol acidification
- Cytoskeleton reorganization
- Production of ROS such as the superoxide anion and H_2O_2 that might have a direct antimicrobial effect as well as contributing to the generation of bioactive fatty acid derivatives and being involved in the cross-linking of cell-wall-bound proline-rich proteins. H_2O_2 can act as a secondary messenger and it is involved in the transcriptional activation of defense genes
- Accumulation of defense-related proteins or pathogenesis related proteins such as chitinases, glucanases and endopolygalacturonases contribute to the release of signaling pectic oligomers (endogenous elicitors), hydroxyproline-rich glycoproteins and protease inhibitors
- Cell death at the infection site (hypersensitive response)

- Structural change in cell wall (lignification of the cell wall, callus deposition
- Transcriptional activation of the corresponding defence-response genes
- Plant defense molecules such as tannins and phytoalexins are detected 2-4 h after stimulation with the elicitor
- Synthesis of jusmonic and salicylic acids as secondary messengers
- Systemic required resistance

However, the study of the chronological order of these events and the interconnection and orchestration between them is complex and still under investigation.

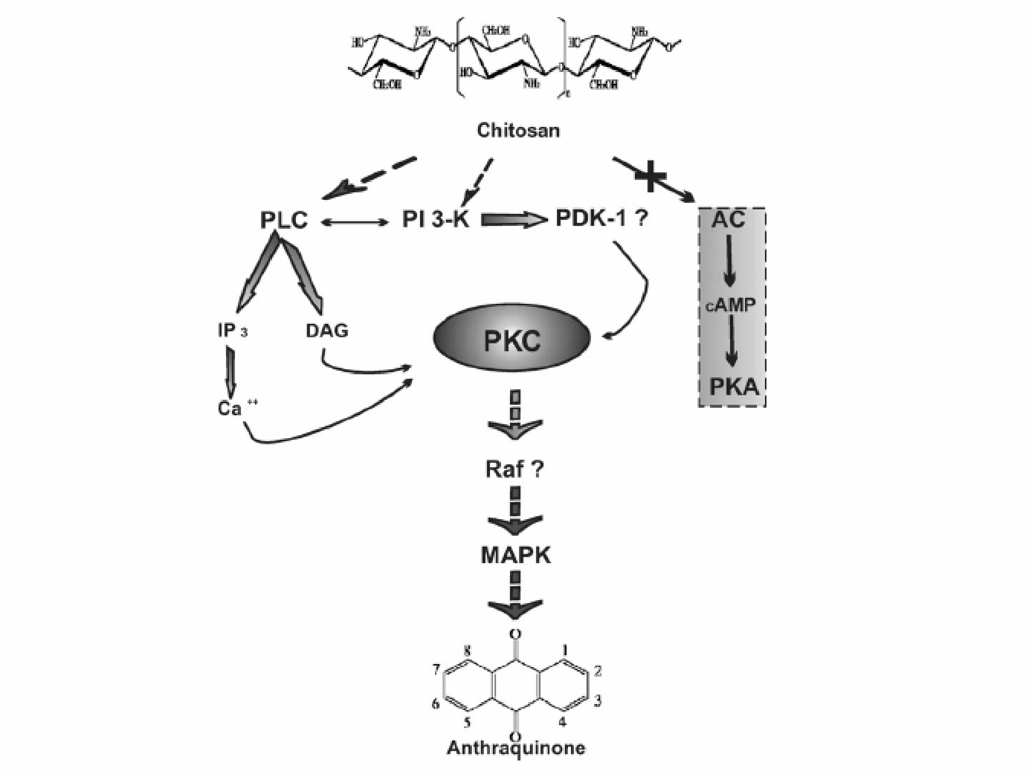

Figure 4 Schematic diagram of signal transduction events involved in the stimulation of anthraquinone by chitosan. The solid arrows indicate strong evidences for the involved pathway; dashed arrows implicate more possible intermediate events not elucidate yet. The cross arrow discard the pathway.

Source : Vasconsuelo *et al*. (2004)

2.2 Elicitors Treatment as a Strategy Production of Secondary Metabolites

Elicitors have been shown to be effective strategy to achieve and increase production of secondary compounds (Dörnenberg and Knorr, 1995). Elicitors have received wide acceptance because of its ability to improve productivity of the plant cell and organ culture (Kim *et al.,* 1997; 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 (Buitelar and Tramper, 1992; Vasconsuelo *et al*, 2005). 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 could have important economical benefits for industry (Radman *et al*., 2003).

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. Different types of elicitors have been used in different systems and given promising results. Some examples are shown in Table 2. Chitosan elicitation, which is the important technique used to enhance the production of several secondary metabolites, has been studied intensively. In case of *P. indica*, it has been shown that chitin or chitosan elicitation could enhance the plumbagin yield by as much as 6.71 times of the basal level (Komaraiah *et al*., 2002).

Although the possibility of enhancing accumulation of secondary products by the addition of elicitors to the medium has been extensively studied in cell cultures, there are only a few reports of elicitors being used in hairy root cultures (Palazón *et al*., 2003).

Table 2 Examples of increased production of phytochemicals, using biotic and abiotic elicitors, in plant cells and tissue culture system of several plant species.

Plant speices	Elicitors	Compounds produced	Increase from non-treated	References
Hairy root cultures				
Ambrosia artemisiifolia	fungal	thiarubrine A	3 folds	Bhagwath and Hjortsø, 2000
Brugmansia candida	yeast extract	scopolamine	~ 7 folds	Pitta-Alvarez *et al*., 2000
		hyoscopolamine	~ 3 folds	
	acetic acid	scopolamine	~ 2 folds	Pitta-Alvarez and Guilietti, 1999
	chitosan	hyoscopolamine	~ 2 folds	
Lithospermum erythrorhizon	fungal	shikonin	30 folds	Brigham *et al*., 1999
Lupinus luteus L.	chitosan	genistein	~ 20 folds	Kneer *et al*., 1999

Table 2 (cont'd.)

Plant speices	Elicitors	Compounds produced	Increase from non-treated	References
Cell suspension cultures				
Arnebia euchroma	fungal mycelium	shikonin	6.15 folds	Fu and Lu, 1999
Catharanthus roseus	fungal mycelium	alkaloid	~ 2-5 folds	Zhao *et al*., 2001
Cistanche deserticola	yeast extract	phenylethanoid	3 folds	Cheng *et al*., 2005
	chaitosan	glycosides	3.4 folds	Cheng *et al*., 2006
Drosophyllum lusitanicum	chitin		~ 1-2 folds	Nahálka *et al*., 1998
Farsetia aegyptia	methyl jusmonate	plumbagin	5 folds	Al-Gendy
	chitosan+methyl jusmonate	glucosinilates	2 folds	and Lockwood, 2005
Plumbago rosea (*indica*) L.	chitosan	plumbagin	6.71 folds	Komaraiah *et al*., 2002
	chitosan	plumbagin	21, 5.7, 2.5 folds	Komaraiah *et al*., 2002
Rubia tinctorum	chitosan	anthraquinone	2 folds	Vasconsuelo *et al*., 2004
Salvia miltiorrhiza	yeast extract	tanhinone	2 folds	Chen and Chen, 2000
Taxus chinensis	chitosan	paclitaxel	2 folds	Luo and He, 2004

3. Hairy Root Culture

The ability of *Agrobacterium rhizogenes* to induce the formation of hairy roots in several dicotyledonous plants was reported in the early 1980's by Chilton *et al.* (1982). *A. rhizogenes*, a soil bacterium, possesses a plasmid called the Ri (root-inducing) plasmid. When plants are wounded, they produce an increased concentration of various phenolic compounds, inducing acetosyringone, which enhances the expression of the virulence (*vir*) genes on the bacterium's Ri plasmid. When the bacterium contacts a plant cell, the *vir* genes encode enzymes that enable the insertion of a segment of the Ri-plasmid (T-DNA) into the genome of plant cell around the wound site (Gelvin, 2000). This T-DNA encodes enzyme that regulates the production of two groups of compounds, the plant growth hormones (i.e., auxins and cytokinins) and unusual amino acids, opine. The plant growth hormones cause the transformed plant cells to form 'hairy' roots, while the opine serve as an exclusive food source for *A. rhizogenes* (Lehninger *et al*., 1994). Hairy root cultures are characterized by a high growth rate and are able to synthesize root derived secondary metabolites. Normally, root cultures need an exogenous phytohormone supply and grow very slowly, resulting in poor or negligible secondary metabolite synthesis (Rao and Ravishankar, 2002).

The advantages of hairy roots are i) exhibit about the same or greater biosynthetic capacity for secondary metabolite production compare to their mother plants (Kim *et al*., 2002) and ii) relatively more stable in secondary metabolites production than cultures of undifferentiated cell, such as callus or cell suspension cultures. The use of hairy root cultures has revolutionized the role of plant tissue culture for secondary metabolite synthesis (Rao and Ravishankar, 2002). Their fast growth, ease of maintenance, and ability to synthesize a range of chemical compounds offer an additional advantage as a continuous source for the production of valuable secondary metabolites. These roots can also synthesize more than a single metabolite and therefore prove economical for commercial production purposes (Giri and Narasu, 2000). The transformed roots of many plant species have been reported for the *in vitro* production of secondary metabolites, for example, solasodine produced from *Solanum aviculare* (Kittipongpatana *et al*., 1998) and higher levels of pulchelin E produced from *Rudbeckia hirta* (Luczkiewicz *et al*., 2002) and plumbagin from *Plumbago zeylanica* (Verma *et al*., 2002).

***In vitro* Production of Plumbagin**

The studies conducted so far could be categorized into two major groups, the micropropagation and *in vitro* synthesis of plumbagin. Workplans are summarized in Table 3 and 4.

Table 3 *In vitro* studies of *Plumbago* spp.

Species	Explant source	Basal medium[1]	Plant growth regulators and other supplements[1]	Response	Reference
P. zeylanica	leaf or stem	MS	BA (4.44 µM)+IAA (1.42 µM)	callus, shoot	Rout *et al.* (1999a)
	node	MS	BA(0.5-1 mg/l)+IAA (0.01 mg/l)	shoot	Rout *et al.* (1999b)
	node	MS	IBA(2.46mµM)+Ads(27.2 mµM)	shoot	Selvakumar *et al.* (2001)
P. indica	leaf	MS	BA (6.7 µM)+IAA (1.4 µM) +Ads (370 µM)	shoot	Das and Rout (2002)
	stem	MS	kinetin (1.5 mg/l)+2,4-D (2.5 mg/l)	callus	Satheesh Kumar and Bhavanandan (1988)
	bud	MS	BA (3 mg/l)	shoot	Chanprame *et al.* (2003)
	hairy root	1/2 B5	--	hairy root	Tatreerod *et al.* (2003)

[1]MS = Murashige-Skoog (1962); B5 = Gamborg *et al*. (1968); BAP or BA = 6-benzylaminopurine or N^6-benzyladenin; NAA = α- naphthaleneacetic acid or 1-naphthaleneacetic acid; IAA = Indol-3-acetic acid; IBA = Indol-3-butyric acid; 2,4-D = 2,4-Dichlorophenoxyacetic acid; Ads= adenine sulfate.

Table 4 Studies on *in vitro* production of plumbagin

Species	Explant source	Basal medium[1]	Plant growth regulator and other supplements[1]	Culture type	Reference
Plumbago zeylanica	young leaf	LS	NAA (0.2 mg/l) +2,4-D (0.2 mg/l)	callus and cell suspension	Choosakul (2000)
Plumbago rosea	young leaf	B5	NAA (1 mg/l)+ kinetin (0.1 mg/l)	root	Panichayupakaranant and Tewtrakul (2002)
	leaf		IAA (1 mg/l)+NAA (0.5 mg/l) +BA (0.3 mg/l)	callus and cell suspension	Komaraiah *et al*. (2001)
Drosophllum lusitanicum	node	Heller	NAA (0.25 mg/l)+IBA(5 mg/l) +BA (0.05 mg//l)	cell suspension	Nahálka *et al*. (1996b)
Dionea muscipula	plants	McC	2,4-D (0.22 mg/l) +NAA (0.18 mg/l)	cell suspension	Hook (2001)

[1]MS = Murashige-Skoog (1962); LS = Linsmaier-Skoog (1965); B5 = Gamborg *et al*. (1968); McC = McCown-Lloyd (1981); BAP or BA = 6-benzylaminopurine or N^6-benzyladenin; NAA = α-naphthaleneacetic acid or 1-naphthaleneacetic acid; IAA = Indol-3-acetic acid; IBA = Indol-3-butyric acid; 2,4-D = 2,4-Dichlorophenoxyacetic acid.

MATERIALS AND METHODS

Materials

1. Chemicals

Standard plumbagin and chitin were purchased from Sigma (USA). The culture media and plant growth regulators were tissue culture grade or equivalent. Extraction solvents were all analytical and/or HPLC grade for plumbagin analysis (Fisher Chemical). Chitosan (T.C. Union food) was a gift from Asst.Prof. Anuwat Jangchat (Faculty of Agro-Industry, KU). Chemicals for immunological study were of high purity grade.

2. Plant materials

The *Plumbago indica* L. plants used for tissue culture work were the *in vitro* grown. Plants of *P. indica* L. which used for immunolocalization studies were collected from field of the Department of Agronomy, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakhon Pathom, Thailand.

3. Apparatus

Rotary shaker, laminar air flow cabinet, vacuum evaporator (Rotavapor® R-200/205 Büchi, Switzerland), HPLC instrument model 1100 series (Agilent Technologies, USA), UV/visible spectrophotometer model Ultrospec 2100 (Amersham Biosciences, England), ultracentrifuge model Optima L-90K (BECKMAN COULTER™ , USA), centrifuge model 7930 (KUBOTA, Japan), sodium dodecyl sulfate-polyacrylamide gel electrophoresis model Mini-PROTEAN®3 cell (BIO-RAD, England), ELISA reader model Multiscan EX version 1.1 (LABSYSTEMS, Finland), microtome model HM 335E (Microm GmbH, Germany) and fluorescence microscope model BX 51(Olympus, Japan)

Methods

1. Quantitative Analysis of Plumbagin in Various Plant Parts and *in vitro* Cultures of *P. indica* L.

Various plant parts (roots, stems and leave) from *in vivo* and *in vitro* grown plants of *P. indica* L. were dried in oven at 50 ^{0}C for 3 days. Dried samples were collected and plumbagin content was determined by high performance liquid chromatography (HPLC) (Choosakul, 2000) with some modifications as described below.

1.1 Preparation of Crude Extracts

Dried samples of various plant parts from *in vivo* and *in vitro* grown plant including hairy roots, calli and cells were grounded to powder with mortar and pestle. Five hundred ml of dried samples were extracted using 20 ml of 100% methanol in 50 ml Erlenmeyer flask placed on an orbital shaker at 100 rpm for 3 days at room temperature. The crude extracts were then filtered with filter paper (Whatman® no. 1).

The culture media from each treatment were extracted with an equal volume of ethyl acetate. These extracts were evaporated at 60 ^{0}C until dried. Each crude extract sample was redissolved with 1 ml petroleum ether and analyzed by HPLC as described by Choosakul (2000) with some modifications described in 1.2.

1.2 Plumbagin Analysis using HPLC

The HPLC system used in this experiment is the Agilent 1100 Series HPLC (Agilent Technologies, USA). Plumbagin was separated on a reversed phase using the Zorbax® SB C18 column (4.6x250 mm, 5 µm particle diameter). Column temperature was maintained at 25 ^{0}C. The mobile phase was run at 1.0 ml/min flow rate using methanol : 0.4 % acetic acid (60:40 v/v). Plumbagin was detected at 270 nm with UV-vis detector. The injection volume for the samples was 10 µl per injection.

Pure plumbagin (Sigma, USA. Catalog no. 481-42-5, lot no. 78H 1550) was used as a standard substance. The linearity of calibration curve was established with a series of solutions prepared by two-fold dilutions of the stock solution with 100% methanol to the final concentrations which ranged from 0.001-10 mg/l (for the samples yielded low plumbagin) and 0.5-4000 mg/l (for the sample yielded high plumbagin). Each concentration was injected in duplicate and the mean value of peak area was taken for the calibration curve with correlation coefficients at 0.99993 and 0.99995.

2. **Immunolocalization of Plumbagin in *Plumbago indica* L.**

Plumbagin is known to accumulate mainly in the roots of *P. indica* L. but their site of accumulation is not known. To understand the pattern of plumbagin accumulation in plant tissue, the polyclonal antibodies were raised against plumbagin-BSA conjugate and used for the immunolocalization of plumbagin.

2.1 Preparation of Plumbagin-BSA Conjugate and The Coating Conjugate

Plumbagin (Sigma, USA. lot no. 78H 1550) was coupled to BSA using glutaraldehyde reaction. The procedures are as described by Chan and Ho (2002) with some modifications stated below.

Plumbagin (0.01g) was disssolved in 100 μl ethanol and was added to BSA (0.07 g) in 10 ml of 1 M phosphate buffer saline (PBS). A total of 0.1 ml of 25% glutaraldehyde was added to the phosphate buffer, making up to a final volume of 10 ml. This latter solution was added dropwise to the protein solution to generate crosslinking of the plumbagin and BSA carrier. Nitrogen gas was bubbled through this solution for 45 min, and the mixture was then constantly stirred under nitrogen at 4 ^{0}C in the dark for overnight. After stirring, the protein conjugate was pelleted at 10,000 rpm for 90 min at 4 ^{0}C, resuspended in PBS, dialyzed exhaustively against 0.5X PBS for 24 h at 4 ^{0}C by changing the buffer 3 times, and stored at -20 ^{0}C until further use.

A conjugate of OVA and plumbagin was also prepared according to the above conjugation protocol using 0.02 g plumbagin and 0.1 g OVA. This plumbagin-OVA conjugate was used as a coating antigen in ELISA.

2.2 Analysis of Plumbagin-Carrier Protein Conjugates

2.2.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The plumbagin-protein conjugates synthesized via the glutaraldehyde reaction were characterized using SDS-PAGE (10% gel). A total of 15 µl each of sample solution (3 µg/ml) were loaded and the gel electrophoresis was run under the voltage of 50 V for 40 min and 100 V for 85 min. The gel was stained in Coomassie staining solution. The migration pattern of these proteins was determined.

2.2.2 Mass Spectrometric Characterization

The mass of plumbagin-BSA conjugate and the standard BSA were determineded by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (BioService Unit, BIOTEC, Thailand Science Park). About 1 pmol of each purified sample was mixed with 9 µl of matrix (10 mg/ml sanapinic acid in 100% acetronitrile containing 0.1 % acetic acid) and sample plate was air-dried. The mass spectra of these proteins were obtained in the linear mode at an accelerating voltage of 1.76 kV. The hapten/protein ratio of each conjugate was determined using the following equation (Chan and Ho, 2002).

Hapten-protein ratio = (conjugate MW- carrier protein MW)/(MW of hapten) : 1

2.3 Polyclonal Antibody Production

A New Zealand White rabbit and BALB/c mice were used for immunizing with plumbagin–BSA conjugate. One mg of plumbagin-BSA conjugate was dissolved in 0.5 ml PBS and mixed with an equal volume of complete Freund's adjuvant. The rabbit was subcutaneously injected with 0.5-1 ml emulsion at the first time, followed by three times of booster injection, at weekly interval, with the initial dose of the conjugate and incomplete Freund's adjuvant. Blood was collected from a marginal ear vein of the rabbit two weeks after injection. The blood samples were allowed to clot for 1 h at room temperature and subsequently, overnight at 4 ^{0}C. The antisera were collected by centrifugation at 10000xg for 10 min at 4 ^{0}C. A 0.02% sodium azide was added to the antisera which were stored at 4 ^{0}C until further use. The antiserum titers were determined by indirect enzyme-linked immunosorbent assay (indirect ELISA) (Jung *et al.*, 2002).

BALB/c mice were injected intraperitoneally with the same antigen using 3 doses; 100, 200 and 400 µg of plumbagin-BSA conjugate. Four times of injections were carried out 4 times at weekly intervals. Blood was collected from the terminal of mouse tails and the antisera were separated as described above.

2.4 ELISA for Anti-Plumbagin-BSA Polyclonal Antibodies

Each well of the 96–well polystylene microtiter plate was coated for 1.5 h at 37 ^{0}C with 50 µl plumbagin-OVA conjugate (5 µg/ml) dissolved in PBS. The plates were then washed three times with PBS containing 0.05% Tween 20 (PBST). The unbound sites of wells were blocked with 200 µl of 8% skim milk and incubated for 2 h at 37 ^{0}C. The plates were washed three times with PBST. The antisera were serially diluted in PBS-8% skim milk from 1:5 to 1: 976563. The diluted samples were added to each well and incubated at 37 ^{0}C for 1.5 h. After incubation, the plates were washed three times with PBST. Fifty microliters of peroxidase-labeled goat anti-rabbit IgG diluted 1: 5000 was added to each well. The plates were incubated at 37 ^{0}C for 1.5 h and then washed three times with PBST. A volume of

100 µl /well of substrate, *p*-nitrophenyl phosphate (PNPP), a concentration of 1 mg/ml was added and incubated for 10 min at 37 ^{0}C. The reaction was stopped by adding 50 µl of 3 M NaOH. Absorbance values at 405 nm were measured using a microplate reader. Control wells of OVA, BSA and normal sera were treated in the same way as test wells (Jung *et al.*, 2002).

The antisera obtained from BALC/c mice were also determined their titer by ELISA as described above but the labeled enzyme was changed from peroxidase-labeled goat anti-rabbit IgG to peroxidase-labeled goat anti-mouse IgG.

2.5 Immunohistochemical Localization of Plumbagin

The rabbit antiserum obtained from the 7th week of the immunization, whose titer was 625, was used for localization of plumbagin in *P. indica* L. plant tissues because its titer was higher than the mouse antiserum. The immunolocalization procedure for detection of plumbagin in plant tissue was modified from Wang *et al*. (1999).

In the experiment, 3-year-old *P. indica* L. roots were fixed by immersion in a cold solution of PBS, pH 7.4 containing 4% (v/v) formaldehyde for overnight at 4^{o}C. The samples were transferred to 25% ethanol in PBS, and serially dehydrated in t-butyl alcohol at the concentrations of 50%, 70%, 85%, 95% and 100%, then replaced with three changes of absolute t-butyl alcohol. The samples were infiltrated and embedded in paraffin. Semi-thin sections (30 µm) were cut with a rotary microtome and affixed to glass slides. The sections were dewaxed using two changes of xylene for 5 min each followed by 100% ethanol and rehydrated by rinsing in water. The sections were blocked in PBS containing 4% skimmilk at 37 ^{0}C for 2 h to reduce nonspecific antibody binding. After washing four times with PBS, sections were incubated with anti-plumbagin-BSA antiserum (diluted 1:10 in blocking solution) for 2 h at 37 ^{0}C. Sections were then washed again with PBS and incubated with fluorescien isothiocyanate (FITC) labeled goat anti-rabbit IgG (ZyMax ^{TM}, USA) (diluted 1:200) for 1 h at room temperature. For control treatments, preimmune serum and anti-BSA antiserum were treated in the same procedures stated above. All samples were visualized with a fluorescent microscope at 495 nm excitation and 525 nm emissions.

3. Establishment of Hairy Root, Callus and Cell Suspension Cultures

3.1 Hairy Root Cultures

Hairy roots cultures were obtained by infecting aseptically stem explants of *P. indica* L. with *Agrobacterium rhizogenes* strain K599, employing the procedure described by Tatreerod (2003). The roots were maintained in hormone-free Murashige and Skoog (1962) (MS) liquid medium with 30 g/l sucrose. They were subcultured in the same medium every 3 weeks and incubated at 25± 2 ^{0}C, in rotary shaker at 100 rpm under dark condition.

3.2 Callus Induction and Cultures

Explants of *P. indica* L. derived from leave, internode and root were placed on MS media supplemented with 0.2 mg/l naphthaleneacetic acid (NAA), 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l kinetin, 30 g/l sucrose and 7 g/l agar for callus induction. Cultures were maintained at 25 ± 2 ^{0}C, 28μmol /m^2/s of 16 h light. Callus formed after two weeks from the explants was then subcultured onto fresh medium every 4 weeks.

3.3 Cell Suspension Cultures

Cell suspension cultures were initiated by inoculation 1 g fresh weight of friable callus into a 125 ml Erlenmeyer flask containing 25 ml of liquid MS medium supplemented with 0.2 mg/l naphthaleneacetic acid (NAA), 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l kinetin, 30 g/l sucrose. The flasks were placed on the rotary shaker at 100 rpm at 25 ± 2 ^{0}C in darkness. Subculturing was done every 3 weeks until a homogenous cell suspension was obtained.

4. Study of Hairy Root, Callus and Cell Suspension Cultures and Plumbagin Production

4.1 The Effect of Basal Media Strength and Sucrose Concentrations on Hairy Root Growth and Plumbagin Production

The hairy root cultures for growth and plumbagin production were performed using different media formulation with two types of basal media: MS (Murashige and Skoog, 1962) and B5 (Gamborg *et al*., 1968) with variation of strengths of basal salts (0.25X, 0.50X, and 1.00X), and the concentrations of sucrose (10, 20 and 30 g/l). The 0.5 g fresh weight (FW) of sterile hairy roots fragments were transferred to a 125 ml Erlenmeyer flask containing 25 ml of liquid medium and shaken at 100 rpm. Hairy roots were grown under the dark conditions at 25± 2 ^{0}C. After 6 weeks of growth, 8 replicate flasks were harvested. The hairy roots and liquid media were separated after filtration. Hairy roots were oven dried at 60 ^{0}C for 3 days and pooled before being analyzed for plumbagin content. Dry growth index (DGI) of hairy root was calculated as described below.

$$\text{Dry Growth Index (DGI)} = \frac{\text{Final Dry Weight} - \text{Initial Dry Weight}}{\text{Initial Dry Weight}}$$

4.2 The Effects of Plant Growth Regulators on Callus Growth and Plumbagin Production

Calli from *in vitro* leaf and internode explants of *P. indica* L. were subcultured on MS salts plus B5 vitamins (MS-B5) supplemented with two concentrations of 2,4-D (0.2 and 0.4 mg/l), two types of cytokinins (BA or kinetin) each at six concentrations range from 0- 0.5 mg/l and the constant amount of NAA (0.2 mg/l). These cultures were grown under condition stated above. The growth of callus was determined at the end of 8 weeks. The dry weight of callus was recorded for DGI calculation and plumbagin content of all five replicates were determined after calli lyophilization for 48 h, using a freeze-dryer at -50 ^{0}C, ground with a mortar and pestle, and pooled before being analyzed by HPLC as describe in section 1. Lateral buds, petiol and roots were also tested aseptically on the best medium for friable callus induction.

4.3 The Effects of Plant Growth Regulators on Cell Suspension Growth and Plumbagin Production

4.3.1 Effects of 2,4-D and Kinetin Concentrations and Light Conditions on Growth and Plumbagin Production

In this experiment, callus tissues obtained from internode and root explants were selected for cell suspension study. The 1 g FW of friable callus tissue was inoculated into 125 ml Erlenmeyer flask containing 25 ml of MS-B5 medium supplemented with 30 g/l sucrose. For cell growth and plumbagin content studies, the above mentioned basal media were supplemented with three concentrations of 2,4-D (0.2, 0.4 and 0.6 mg/l), five concentrations of kinetin (0.1- 0.5 mg/l) and the constant amount of NAA (0.2 mg/l). The cultures were shaken at 100 rpm and grown both in the dark and in the light condition of 16 h light intensity at 55 μmol /m ^{2}/s at 25 ± 2^{0}C. The growth of cell was determined at the end of 3 weeks. In each medium, cells from all ten replicates were harvested by vacuum filtering of the culture medium through filter paper. The dry growth index (DGI) of cell was calculated after the cells were oven dried at 60 ^{0}C. The dried cells were pooled and ground into powder before being analyzed for plumbagin content. Culture media were also extracted and analyzed by HPLC as described in section 1.

4.3.2 Relationship between Cell Growth and Plumbagin Production in Cell Suspension Cultures

Cell culture derived from *in vitro*-root-derived friable callus was maintained on MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 0.5 mg/l kinetin and 30 g/l sucrose. Subculturing was done every 3 weeks until a homogeneous cell suspension was obtained. For the experiment, 1g FW of homogeneous cell suspension was transferred to each 125 ml Erlenmeyer flask containing 25 ml liquid medium stated above. The culltures were shaken at 100 rpm and were grown under the dark condition at 25 ± 2 ^{0}C. Each treatment will be done in ten replicates. The DGI of cell suspension was determined at different ages of 3, 6, 9, 12, 15, 18, 21, 24 and 27 day-after-culture. The plumbagin content was also measured using HPLC as described in section 1.

5. Effects of Biotic and Abiotic Elicitation of Plumbagin in Hairy Root and Cell Suspension Cultures

5.1 Preparation of Elicitors

5.1.1 Chitosan

Chitosan (94.68% deacetylation, obtained from T.C. Union Foods Co, Ltd., Thailand) was prepared according to Komaraiah *et al*. (2003). One g chitosan was dissolved in 2 ml of glacial acetic acid by adding drop wise at 60 ^{0}C for a period of 15 min and the final volume was made up to 100 ml with deionized water. The pH of the solution was adjusted at 5.7 with KOH prior to use as an elicitor.

5.1.2 Chitin

Chitin powder from crab shells (Sigma, USA. lot. no. 215 7443) was prepared by weighting at various concentrations, put in each tube and autoclaved before used as an elicitor.

5.1.3 Fungal Elicitor

The mycelia of *Colletotrichum capsici* were grown in 125 ml Erlenmeyer flasks containing 25 ml of Czapek Dox medium. Cultures were incubated at 28 ^{0}C on shaker at 200 rpm for 3 weeks. Fully-grown mycelia were collected by filtration through cheesecloth and washed twice with deionized water, and then homogenized in liquid nitrogen with a pestle and mortar. The mycelial homogenate was autoclaved at 121 ^{0}C for15 min and oven dried at 60 ^{0}C for 2 h and was weighted to the amount used in each treatment.

5.1.4 Yeast Elicitor

Yeast extract was separated into two types, non-precipitate and precipitated with methanol. For the non-precipitate type, yeast extract was weight and dissolved in deionized water and autoclaved at 121 ^{0}C for 15 min and final concentrations of 50-300 mg/l were used in the experiment. For the methanol precipitation type, carbohydrate fraction isolated from the yeast extract was prepared as described by Komaraiah *et al.* (2003) with some modifications. Ten g yeast extract was dissolved in 100 ml of distilled water. Methanol was added to 80 % (v/v). After stirring for 3 days at 4 ^{0}C, the solution was centrifuged at 10000xg for 20 min. For stock solution, the pellet was collected and re-dissolved with deionized water and adjusted the concentration to 1,000 mg/l, autoclaved at 121 ^{0}C for 15 min.

5.2 Effects of Concentrations and Exposure Time to Elicitors in Hairy Root Cultures of *P. indica* L.

The hairy root cultures were maintained on hormone-free 1/2 MS liquid medium supplemented with 20 g/l sucrose. The culture was incubated at 25± 2 ^{0}C, in rotary shakers at 100 rpm in darkness and was subcultured in the same medium every 3 weeks for elicitation experiments.

5.2.1 Elicitation with Chitin

Five hundred mg fresh weight of 21-day-old hairy roots of *P. indica* L. were transferred to 25 ml of 3 formulas of liquid medium (1/4 MS, 1/2 MS and 1/2 B5) supplemented with 20 g/l sucrose. Hairy roots were maintained until 21 days and elicited with chitin. To study the effects of concentrations of elicitor, five concentrations (100, 200, 300, 400 and 500 mg/l) of chitin were added to 21-day-old hairy root cultures in 25 ml of each media in 125 ml Erlenmeyer flask. Sterilized water was used as control. The incubation condition was described above. Ten flasks were used for each condition tested. DGI and plumbagin accumulated in hairy roots and released into the medium were determined.

5.2.2 Elicitation with Chitosan

Five hundred mg fresh weight of 21-day-old hairy roots of *P. indica* L. were transferred to 25 ml of 3 formulas of liquid medium (1/4 MS, 1/2 MS and 1/2 B5) supplemented with 20 g/l sucrose, contained in 125 ml Erlenmeyer flasks. Hairy roots were maintained until 21 days and elicited with chitosan. Final chitosan concentrations in hairy root cultures were 100, 200, 300, 400 and 500 mg/l. Final acetic acid concentration 0.1%, which was a high level of acetic acid employed as control. The pH of media was adjusted to 5.7 with KOH before autoclaved. The incubation conditions are described above. Ten flasks were used for each condition tested. Dry growth index (DGI) and plumbagin accumulation in the roots and release into the medium were determined. The higher concentrations of chitosan (500, 1,000, 1,500, 2,000 and 2,500 mg/l) were also tested in hairy root culture as described the experiment conditions above.

5.2.3 Elicitation with Other Elicitors

The effects of concentration of acetic acid, fungal elicitor and yeast elicitor (non-precipitated) on growth and the production of plumbagin from hairy roots were also studied. The concentrations used in the study were listed in Table 5. Elicitors were added to the 21-day-old cultures. In all elicitation, hairy roots and culture media were harvested after 5 days of treatment and analyzed for plumbagin content using HPLC.

Table 5 Concentrations of elicitors used in the study of the effect of concentration in hairy root cultures.

Elicitor	Type	Concentration (mg/l)
acetic acid	abiotic	0, 0.02, 0.04, 0.06, 0.08 and 0.1 (%)
precipitated yeast extract	biotic	0, 50, 100, 150, 200
fungal mycelium	biotic	0, 50, 100, 150, 200

5.2.4 Effects of Time Exposure to Elicitors in Hairy Root Cultures

According to the experiment in section 5.2.1 and 5.2.2, chitin and chitosan which were good elicitors to improve the plumbagin production and promote the released of plumbagin into culture media were selected for time of exposure studied.

The effect of time exposure to chitin was studied on 21-day-old hairy root cultures in 1/2 MS and 1/2 B5 liquid media challenged with 400 mg/l chitin. The cultures were incubated in darkness at 25 $\pm$ 2 ^{0}C and shaken at 100 rpm. Hairy root cultures were harvested at 8, 9, 10 and 11 days of treatment. In each period, hairy root cultures were not shaken for 6 h before harvesting. DGI was calculated and plumbagin content was determined by HPLC.

The similar experiment was conduct using chitosan as elicitor. The concentration of chitosan was 300 mg/l. Hairy root cultures were harvested at 1, 2, 3, 4, 5, 6 and 7 days after treated with chitosan. The DGI was calculated and plumbagin content determined.

5.3 Effects of Concentrations and Exposure Time to Elicitors in Cell Suspension Cultures of *P. indica* L.

5.3.1 Effects of Elicitors Concentration

One g fresh weight of 12 day-old-cell suspension cultures of *P. indica* L. was transferred to 25 ml of MS-B5 supplemented with 0.2 mg/l 1 NAA, 0.2 mg/l, 2,4-D, 0.5 mg/l, kinetin and 30 g/l sucrose, in 125 ml Erlenmeyer flasks. Cell suspension cultures were maintained until 12 days and elicited with elicitors. In all treatments, cultures were incubated in darkness at 25$\pm$ 2 ^{0}C.

a. Elicitation with Chitin

Chitin was added to 12-day-old cell suspension cultures of *P. indica* L. at concentrations of 100, 200, 300, 400 and 500 mg/l in 25 ml of liquid media as described above. Sterilized water was used as the control treatment. The incubation conditions are described above. Ten flasks were used for each condition tested. DGI and plumbagin content in hairy roots or cells and release into the medium were determined.

b. Elicitation with Chitosan

Final chitosan concentrations in cell suspension cultures were 100, 200, 300, 400 and 500 mg/l. Two controls were used: one without acetic acid (sterilized water) and the other with 0.1% acetic acid which was a highest level of acetic acid employed. The pH of media was adjusted at 5.7 with KOH before autoclaved. The incubation conditions were described as above. Ten flasks were used for each condition tested. DGI and plumbagin accumulation in the cell and culture media were determined.

c. Elicitation with Other Elicitors

The effects of concentration to fungal elicitor, two types of yeast elicitor (precipitated and non-precipitated with methanol) on growth and the production of plumbagin from cell suspension cultures were also studied. The concentrations used in the study were shown in Table 6. For each treatment, elicitors were added to the 12-day-old cell cultures. Cell suspension cultures were harvested after 5 days of treatment and analyzed for plumbagin content as described in session 1.

Table 6 Concentrations of elicitors used in the study of the effect of concentration in cell suspension cultures

Elicitor	Type	Concentrations (mg/l)
precipitated yeast extract	biotic	0, 50, 100, 150, 200, 250, 300
yeast extract	biotic	0, 50, 100, 150, 200, 250, 300
fungal mycelium	biotic	0, 100, 200, 300, 400, 500

5.3.2 Detection of Cell Death after Elicited with Chitin or Chitosan

One ml of cell suspension cultures after elicitation with chitin or chitosan in each concentration was incubated for 10 min with 0.05% Evan’s blue and washed with 80 ml deionized water to remove excess and unbounded dye. Dye bounded to dead cells was then solubilized in 50% methanol with 1% sodium dodecyl sulfate (SDS) for 30 min at 50 ^{0}C. Cell suspension culture which was not incubation with Evan’s blue was used as control. Cells treatments were measured the absorbance at 600 nm (Qin and Lan, 2004).

5.3.3 Effects of Exposure Time to Elicitors

The effect of exposure time was studied on 12-day-old cell suspension cultures in media and conditions stated in 3.3.1 and challenged with 300 mg/l chitin or 200 mg/l chitosan. The cell suspension cultures were harvested at

1, 2, 3, 4, 5, 6 and 7 day-after-elicitation. DGI was calculated and plumbagin content was determined.

5.4 Plumbagin Released into Liquid Medium of *P. indica* L. Hairy Root and Cell Suspension Culture by Multiple Elicitation with Chitosan

The 21-day-old hairy root cultures were repeatedly treated with 300 mg/l chitosan in 1/2 MS and 1/2 B5 liquid medium supplemented with 20 g/l sucrose. Plumbagin content was analyzed during the time course of hairy root culture. Five replication flasks were used in all experiments.

In case of cell suspension, 12-day-old culture was repeatedly treated with 200 mg/l chitosan in MS-B5 medium supplemented with 0.2 mg/l 2,4-D, 0.5 mg/l kinetin and constant NAA (0.2 mg/l) with 20 g/l sucrose. Plumbagin content was analyzed during the time course of cell suspension culture.

RESULTS AND DISCUSSION

1. Quantitative Analysis of Plumbagin in Various Plant Parts and *in vitro* Cultures of *P. indica* L.

1.1 Standard Calibration Curve

Two standard calibration curves for HPLC analysis were prepared as described by Choosakul (2000) and used in the experiments. The first one is the calibration curve of standard plumbagin showing linearity of the relationship from 0.001-10 mg/l and the correlation coefficient was 0.99993 to be used for the samples that yielded low plumbagin content. The second one is the calibration curve of standard plumbagin showing linearity of the relationship from 0.5-4000 mg/l and the correlation coefficient was 0.99995 to be used for the sample that yielded high plumbagin content (Figure 5). The chromatogram of standard plumbagin revealed the retention time of approximately 13 and 15 min (Figure 6A, 6C).

1.2 Plumbagin Content in Various *In Vitro* Grown *P. indica* L. Plant

Plumbagin was observed at various concentrations in whole *P. indica* L. plant both *in vivo* and *in vitro*. For the *in vivo* plant, 7.17 mg/g DW in roots, 0.11 mg/g DW in stems and 0.003 mg/g DW in leaves were detected. The plumbagin level present in *in vitro* plant was; 2.45 mg/g DW in roots, 0.5 mg/g DW in stems and 0.02 mg/g DW in leaves as shown in Figure 7. Therefore, roots are confirmed as a potential source of plumbagin. This result also confirms the previous report of plumbagin content obtained from *in vivo* plant of *P. zeylanica* L. which is a close relative to *P. indica* L. The plumbagin content in roots appeared the highest followed by the stems and leaves, respectively (Choosakul, 2000).

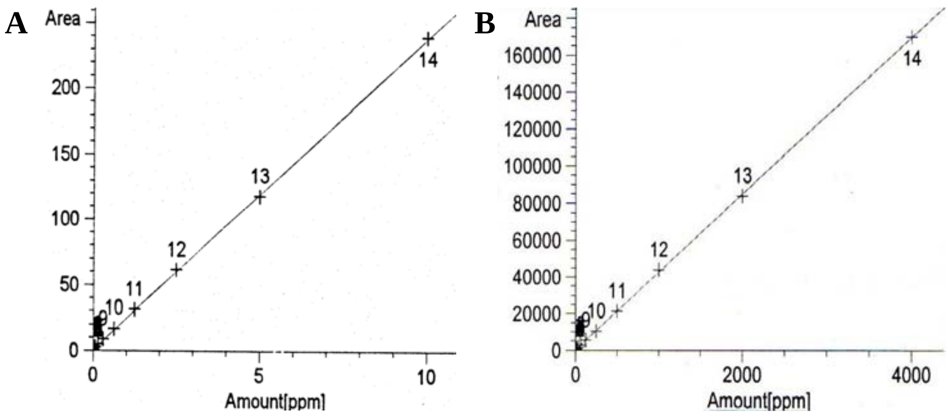

Figure 5 Calibration curves of standard plumbagin for HPLC analysis

A) The linearity of the relationship from 0.001-10 mg/l

B) The linearity of the relationship from 0.5-4000 mg/l

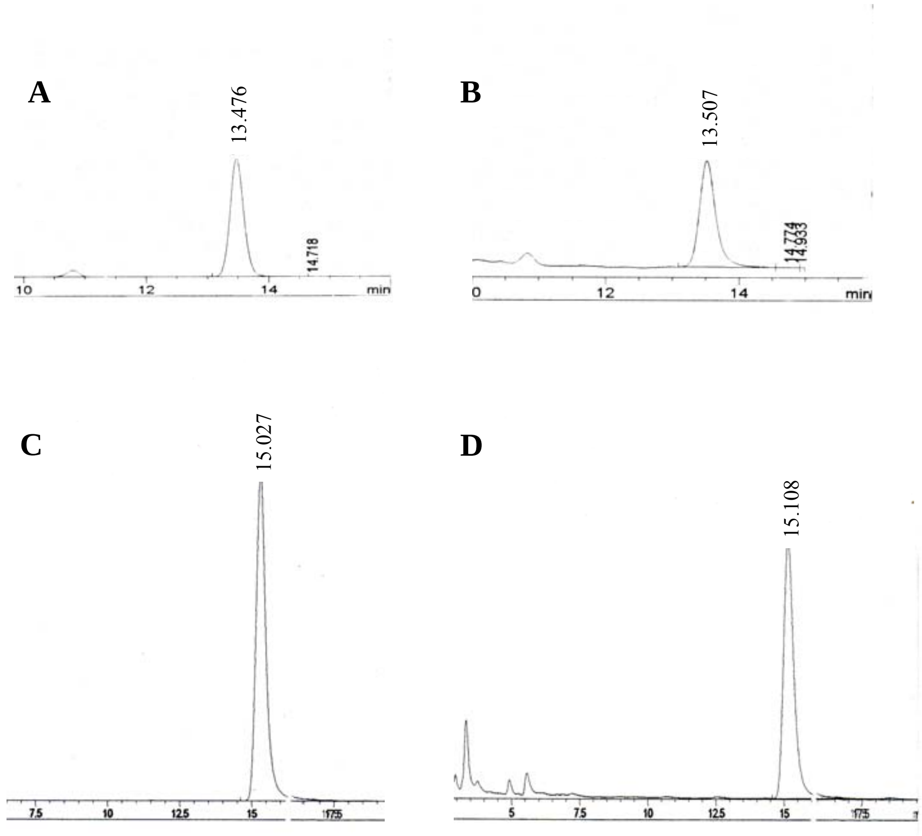

Figure 6 The HPLC chromatograms of plumbagin standard (Sigma, lot no. 78H 1550) (A and C), plumbagin in callus (B) and plumbagin in hairy root (D)

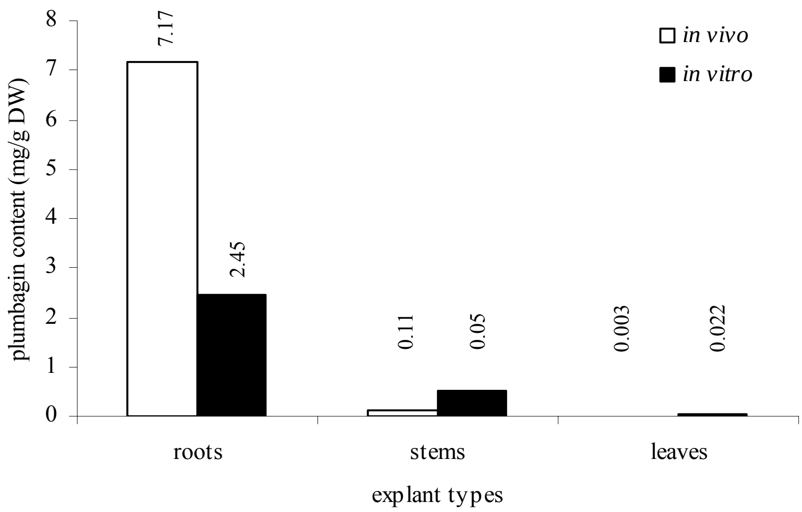

Figure 7 Plumbagin content in various parts of *in vivo* and *in vitro* *Plumbago indica* L.

2. **Immunolocalization of Plumbagin in *Plumbago indica* L.**

2.1 Preparation of Plumbagin-Carrier Protein Conjugates

To prepare plumbagin-protein conjugate, the plumbagin: BSA ratio of 50:1 was calculated based on their molecular weights. The conjugation was carried out by glutaraldehyde reaction (Chan and Ho, 2002). At the beginning, the color of solution was changed from dark yellow to yellow. After stirring under nitrogen gas, the color of solution changed from yellow to green (Figure 8). At the final stage of conjugation, the plumbagin-BSA conjugate was obtained. However, no color change was observed with the mixture solution of plumbagin-OVA according to the same procedure.

2.2 Analysis of Plumbagin-Carrier Protein Conjugates

2.2.1 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In order to confirm the success of plumbagin-protein conjugation by glutaraldehyde reaction, SDS-PAGE was employed. The results shown in figure 9 were native BSA and OVA, plumbagin-BSA and plumbagin-OVA conjugates. The bands clearly indicated that hapten (plumbagin) was coupled to BSA or OVA according to the higher molecular weight bands of the conjugates than the native proteins. The plumbagin-BSA conjugate band had higher molecular weight than the plumbagin-OVA conjugate. BSA is easy to dissolve and often used as a carrier for most purposes. Although OVA is difficult to dissolve, it is a good choice for antigen coating in ELISA instead of plumbagin-BSA in order to eliminate the cross reaction with the anti-BSA in the serum (Harlow and Lane, 1988; Hermanson, 1996). No band of plumbagin alone appeared on the gel when plumbagin was loaded.

2.2.2 Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

The MALDI-TOF MS revealed the molecular weight of native BSA and plumbagin-BSA conjugate to be 66564.232 Da and 72817.704 Da respectively, as indicated in Figure 10. This technique demonstrated the greater resolution in molecular weight determination compared to SDS-PAGE.

One previous study reported the successfully raised polyclonal antibodies to recognize the 1,2-naphthoquinone and developed immunostaining to detect protein modification by 1,2-naphthoquinone in animal cell (Zheng and Hammock, 1996). Since the plumbagin is categorized in the same group of naphthoquinone, the immunoassay is thought to be a useful tool in detection of this substance in plant cell. To prepare an immunogen, plumbagin, a low molecular weight hapten (MW 188.2), was conjugated with BSA by glutaraldehyde reaction. The conjugate were analysed with MALDI-TOF MS which is well known as a powerful tool for analysis of a wide range of biomolecules, such as peptide and proteins (Prasain *et al*., 2004) and hapten-protein conjugates (Adamczyk *et al*., 1996). Therefore, this technique was used for the characterization of plumbagin-BSA conjugate and native BSA.

In contrast to SDS-PAGE, MALDI-TOF mass spectrometry allows accurate determination of molecular weight of the protein studied. From the results (Figure 10), the molecular weight of standard BSA was determined at 66564.232 Da similar to that previuosly reported (66432.9 Da) by Jirayama *et al.* (1990) corresponding to an accuracy of 99.80%. MALDI-TOF MS shown in this study was suitable technique for providing direct evidence of hapten-protein conjugation, and for estimating the hapten/protein ratios of the synthesized immunogens. MALDI-TOF MS is the most suitable only for the molecular weight ranged between 0.4 and 400 kDa (Chan and Ho, 2002). Based on the respective molecular weight of plumbagin;

188.2 Da, the calculated hapten/protein ratio for plumbagin-BSA conjugate was determined to be 33:1. The result showed that this ratio was sufficient for eliciting antibody response in rabbit.

Figure 8 The color change during the conjugation procedure.

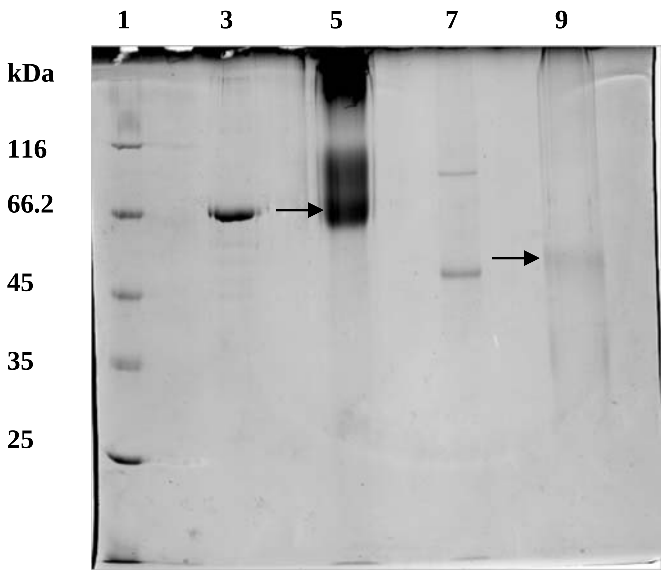

Figure 9 Analysis of plumbagin-protein conjugates using SDS-PAGE

lane 1, protein markers (4 µl); lane 3, native BSA (3 µg/ml, 15 µl);
lane 5, plumbagin-BSA conjugate (3 µg/ml, 15 µl);
lane 7, native OVA (3 µg/ml, 15 µl) and
lane 9, plumbagin-OVA conjugate (3 µg/ml, 15 µl).

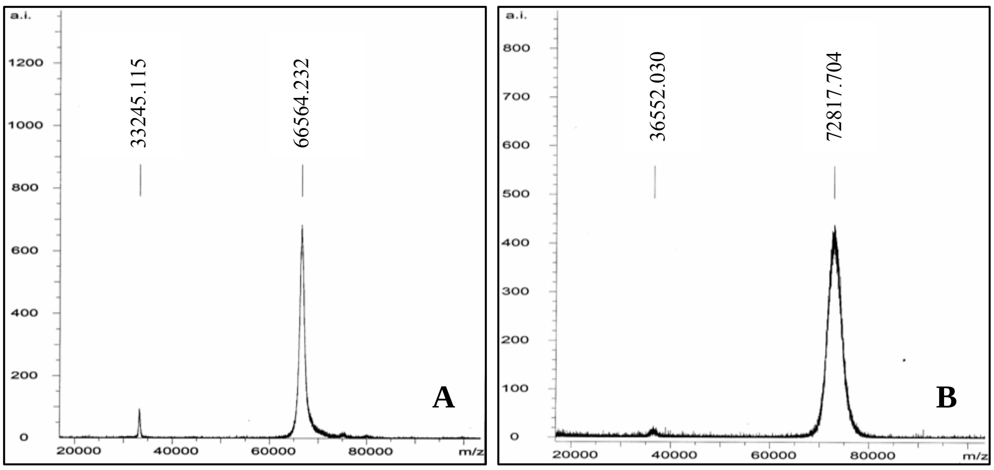

Figure 10 MALDI-TOF mass spectra of samples from plumbagin-carrier protein conjugates, (A) = native BSA, (B) = plumbagin - BSA conjugate

2.3 Polyclonal Antibody Production and ELISA Determination

The conjugate specific polyclonal antibodies are raised in rabbits and BALC/c mice. The antiserum titers were determined by enzyme-linked immunosorbent assay (ELISA). In preliminary test, the mouse antisera were obtained 2 weeks after the fourth immunization. The titer was 25 which was obtained from the concentration of 100 mg/l plumbagin-BSA conjugat. The tested antisera yielded low a titer in all concentrations of the conjugate. Due to mouse antisera had low titer and not enough volume for localization of plumbagin in *P. indica* L. tissues. Therefore, rabbit antiserum was used for further experiment.

The rabbit antisera were obtained 2 weeks after the first immunization and the titers were presented in Figure 11. The highest titer was 625 which was obtained on the 7th week of blood collection.

To avoid the cross reactivity in ELISA, plumbagin-OVA conjugate was used as a coating antigen which produced the OD_{405} of 1.693 when reacted with anti-plumbagin-BSA antiserum. The plumbagin-OVA conjugate showed a better binding efficiency to the ELISA plate than plumbagin alone (OD_{405} 0.072). The result indicated that the immunogen was able to elicit specific antibody recognizing the plumbagin. No reaction was observed when hapten-BSA, native BSA and OVA were allowed to react with the preimmune sera.

In summary, the production of rabbit antisera could be accomplished although the obtained titers were very low.

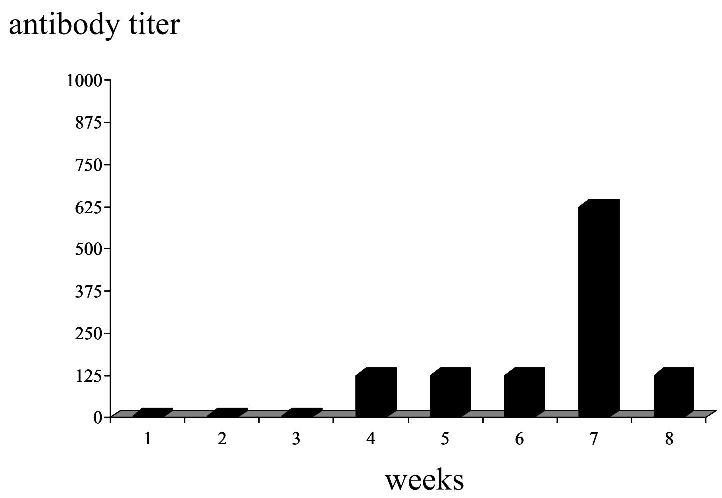

Figure 11 Antibody titers of the rabbit antisera raised against plumbagin-BSA conjugate determined by ELISA.

2.4 Immunohistochemical Localization of Plumbagin

In order to localize the plumbagin in plant tissues by immunofluoresent technique, the rabbit antiserum against plumbagin conjugate was prepared. The antiserum was applied to microscopic sections of *P. indica* L. root, stem and leaf. The result from the immunolocalization experiment confirmed the specific reaction of the antigen–antibody complex established in ELISA. Eventhough polyclonal antibody had low titer, the reaction indicated that immunohistochemical techniques can be used to determine the localization of plumbagin at tissue and cellular levels by fluorescent microscopy. The results demonstrated that plumbagin was found to accumulate in all parts of *P. indica* L. plant but mainly in root as seen by the intense green fluorescence (Figure 12). However, the main area of accumulation was found in epidermal cells and cortex tissues but not in vacuole (Figure 13) and vascular tissue (Figure 14). No reaction was observed when treated the tissue with anti-BSA or preimmune sera (Figure 13).

To determine which tissues, cells or cellular components of *P. indica* L. contain plumbagin, the development of a modified tissue-processing protocol is required. The immunohistochemical techniques have to be able to limit the migration of plumbagin because it is well soluble in alcohol, acetone, chloroform, benzene and acetic acid. Immunolocalization of plumbagin presents a number of technical problems, especially those related to the leaching of the antigen during chemical fixation and the rapid dehydration of the plant materials. The tissue preparation methods that prevent the diffusion of secondary metabolite is crucial. The immunohistochemical assay using low temperature preparation techniques have been successfully used for localization of phytohormones, the low molecular weight substances in plant tissues (Zavala and Brandon, 1983). Therefore, the modification of tissue fixation using low temperature for tissues preparation coupled with immunohistochemical assay for localization of plumbagin were employed in this experiment.

However, the low resolution of the fluorescent microscope did not permit the recognition of plumbagin in subcellular level. For further exploration, the immunoelectron microscopic technique by utilizing immunogold-labeled antibody may be applied. This technique will increase the possibility to recognize a number of subcellular compartments believed to be involved in the biosynthesis and accumulation of secondary metabolites.

The plumbagin is a low molecular weight and there has not been report about the key enzyme which involved the biosynthetic pathway of plumbagin. It could be suggest that finding the biosynthetic enzymes which involved the plumbagin synthesis is usefull for antibody production against plumbagin. Due to the enzyme might be yield higher titer than using plumbagin alone. The localization of secondary metabolites mainly studied the enzymes involved in biosynthetic pathways such as flavonoid enzyme in *Arabidopsis* roots (Saslowsky and Shirley, 2001); homospermidine synthase in alkaloids biosynthesis in *Eupatorium cannabinum* (Anke *et al*., 2004); and phenylalanine ammonia-lyase in leaves of *Phyllanthus tenellus* (Santiago et al., 2000).

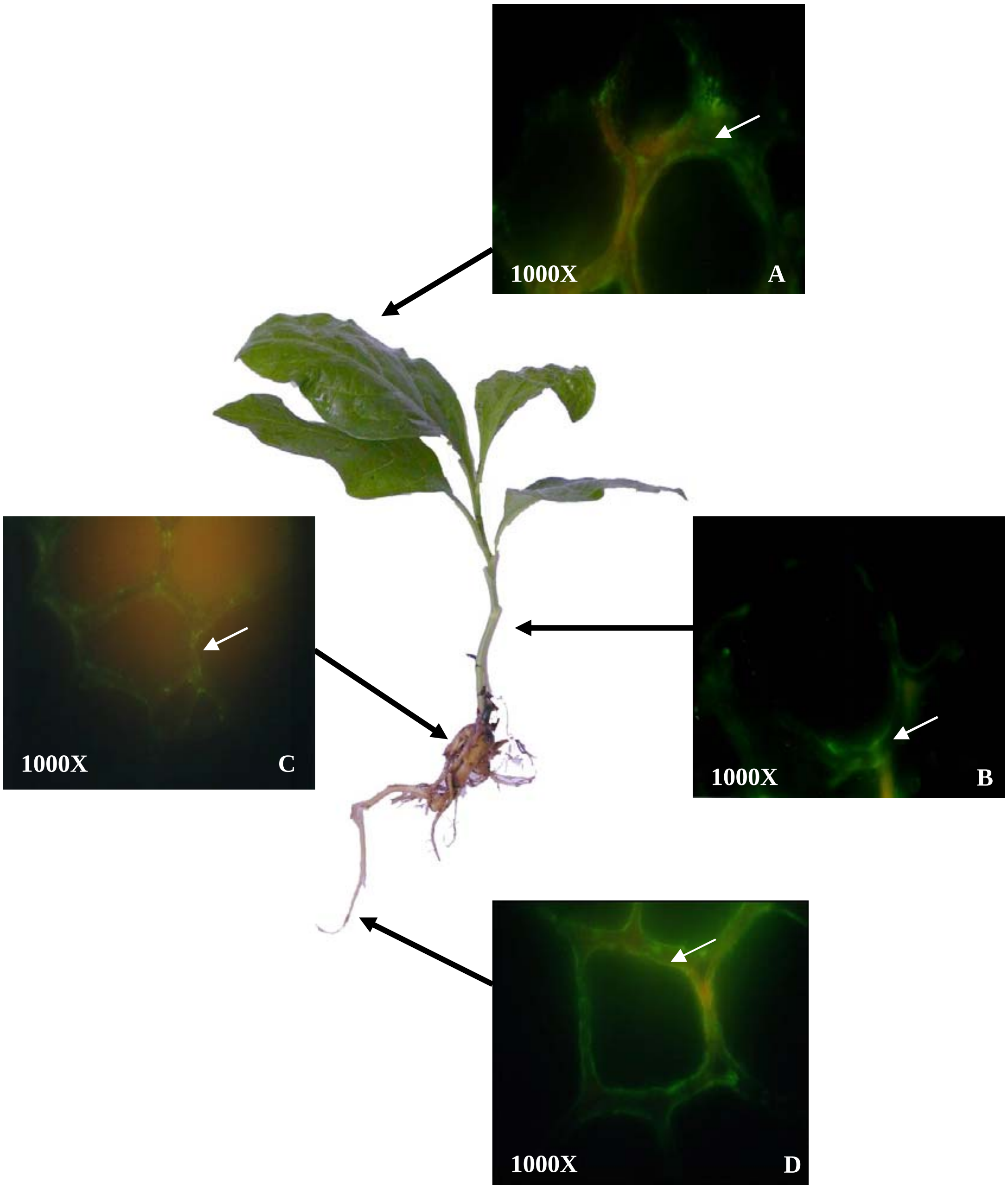

Figure 12 Immunolocalization of plumbagin in cross sections of *Plumbago indica* L. plant parts (collected in November) using fluorescein isothiocyanate (FITC) labeled secondary antibody; leaf (A); stem (B); upper part of root (C) and lower part of root (D)

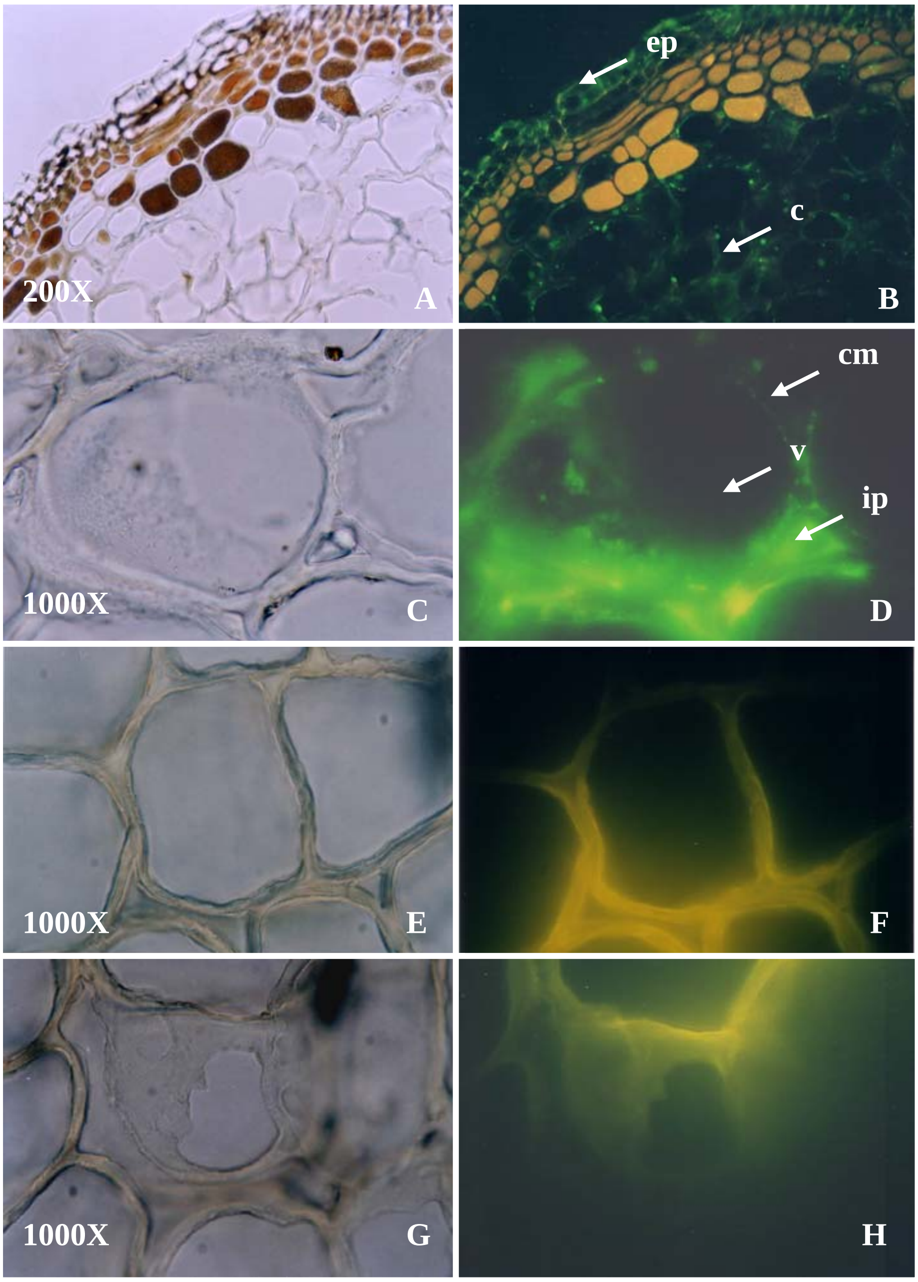

Figure 13 Immunolocalization of plumbagin in cross sections of 3-year-old *Plumbago indica* L. root using fluorescein isothiocyanate (FITC) labeled secondary antibody. Root sections incubated with anti- plumbagin BSA conjugate antibody (B and D); control section treated with pre-immuned serum (F) and with anti-BSA (H) were explored using fluorescent microscope. Light microscopic pictures of each sample were shown in Figure A, C, E, and G. The abbreviations; ep (epidermal cell), c (cortex), v (vacuole) ip (intercellular space) and m (membrane)

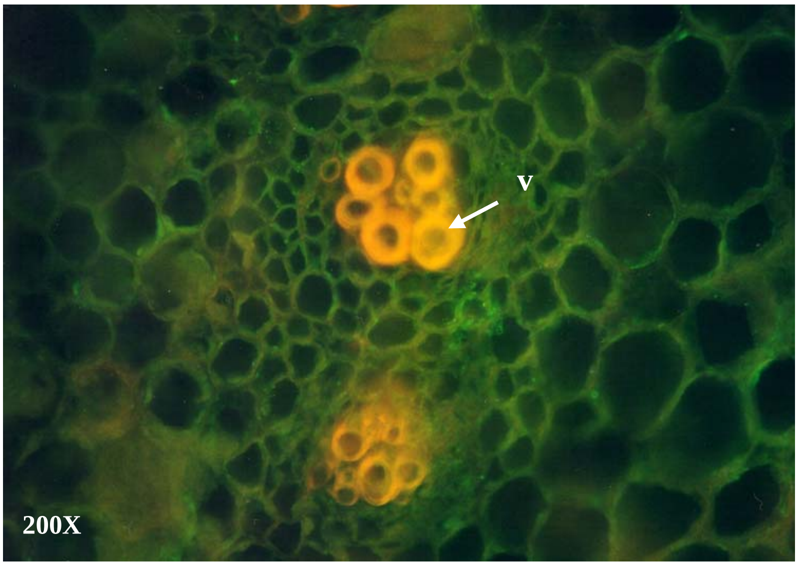

Figure 14 Immunolocalization of plumbagin in cross sections of *Plumbago indica* L. leaf using fluorescein isothiocyanate labeled secondary antibody.
The abbreviation; v (vascular tissues)

3. <u>Establishment of Hairy Root, Callus and Cell Suspension Cultures and Growth Study</u>

3.1 The Effect of Basal Media Strength and Sucrose Concentrations on Hairy Root Growth

The hairy root lines which induced by *Agrobacterium rhizogenese* strain K599 as described by Tatreerod (2003) was used throughout this study. Among two liquid media formulas tested, the DGI of hairy roots grown in MS basal media were higher than those of in B5 basal media, especially in 1/2 MS liquid medium. The 1/4 B5 liquid media yielded the poorest hairy root growth. Regardless of media formula, the order of the increasing of DGI among hairy roots culture in different media strength was 1/2 x, 1x and 1/4 x (Figure 15). The similar results reported that the hairy root culture of *Solanum laciniatum* Ait. grew on half-strengh MS medium better than on full-strengh MS (3% sucrose) (Okršlar *et al*., 2002). The hairy root culture of *Atropa baetica* also yielded the hightest dry weigh in a half-strength MS medium than in a full-strength MS medium (Zárate, 1999). However, these results were considered only in the treatments in which 30 g/l sucrose were added. There was no significant in the growth of hairy roots in all strength of MS basal media containing with 20 g/l sucrose.

Sucrose concentration is known to affect a range of culture parameters such as growth, primary metabolism and yield of secondary products. In general, growth rate is considered as a function of sucrose concentration (Jacob and Malpathak, 2004). In this study, four different concentrations of sucrose were tested. The DGI of hairy roots was increased with increasing sucrose concentrations within the range of 0-20 g/l and was decreased at the concentration of 30 mg/l when B5 media was used. However, it was increase thoroughly in the sucrose concentrations range of 0-30 g/l in MS media (Figure 15 and 16). With no sugar added into the media, the hairy root growth was limited in all treatments. The highest DGI of hairy roots obtained from 1/2 MS liquid medium supplemented with 30 g/l sucrose was 8.64 , and the highest DGI of hairy roots obtained from 1/2 B5 liquid medium supplemented with 20 g/l sucrose was 7.13 (Figure 15). The sucrose concentration clearly affected the growth of *P. indica* L. hairy root. The similar result in hairy root of *Solanum aviculare* Forst was reported. The strengths of the medium and concentrations of sucrose played crucial roles in the growth rate of those hairy root cultures (Kittipongpatana, 1998).

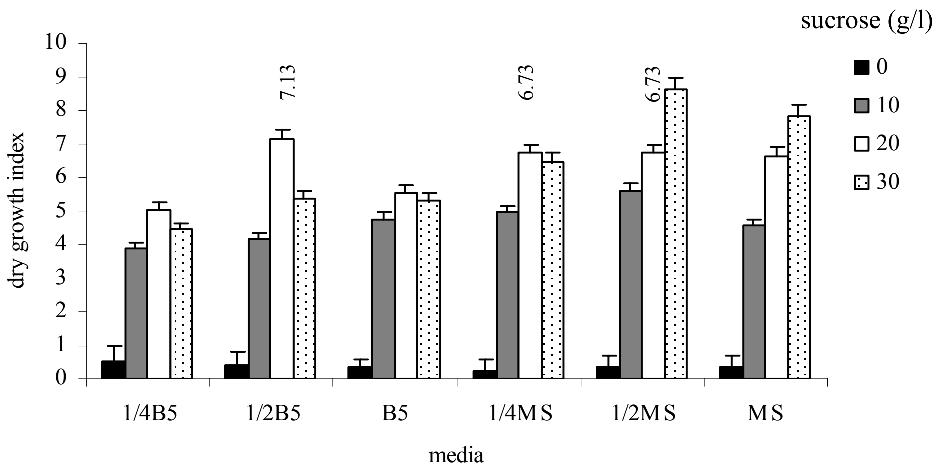

Figure 15 The dry growth index (DGI) of hairy roots on different liquid media and different sucrose concentrations after culture at 25±2°C, 55 μmol/m²/s 16 h light, for 6 weeks. The initial dry weight in all cases was 0.04 g. The results represent means of eight replications.

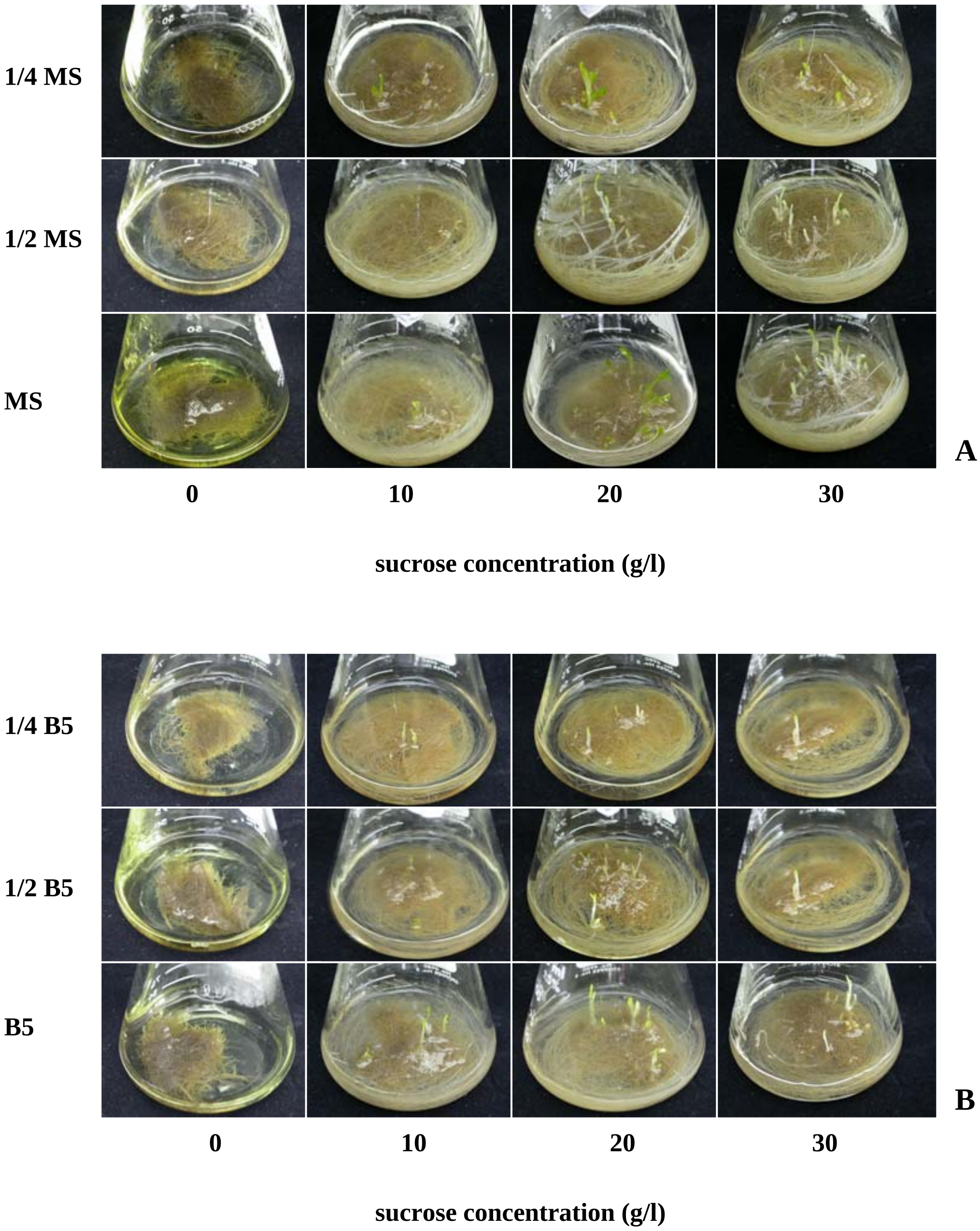

Figure 16 Hairy roots of *Plumbago indica* L. cultured in different liquid media and different sucrose concentrations after culturing at 25±2°C, 55 µmol/m^2/s 16 h light, for 6 weeks.

A) MS medium

B) B5 medium

3.2 Effects of Auxin and Cytokinin on Growth of Callus

The small, compact and slow growing callus was preliminary observed in leaf explants cultured in MS medium supplemented with 2,4-D alone, or in concerted with BA or NAA. Thus, for the subsequent experiments, calli were initiated from leaf and internode explants using MS-B5 medium supplemented with various concentrations of BA (0-0.5 mg/l) or kinetin (0-0.5 mg/l) in combination with 2,4-D (0.2-0.4 mg/l) and a constant amount of NAA (0.2 mg/l). The leaf explants enlarged and calli developed on the cut surface after 7-10 days while the internode explants developed friable callus which covered the entire surface of the explant within 4 weeks. The calli were green at the beginning and turned dark gray after 3-4 weeks. These results were similar to the previous reports in callus cultures of *Plumbago rosea* (Satheesh Kumar and Bhavanandan, 1988) and *Drosophyllum lusitanicum* (Budzianowski *et al*., 2002).

In comparison, the leaf explant yielded better callus formation than that of the internode explant. The calli established on several media could be divided into two types, compact and friable callus. For the leaf explant, the friable calli were obtained from MS-B5 medium supplemented with 0.2 mg/l NAA and all various concentrations of kinetin in combination with 2,4-D or supplemented with 0.2 mg/l NAA, 0.2 or 0.4 mg/l 2,4-D and 0.1 mg/l BA. The highest dry weight of callus was obtained from leaf explants induced on MS-B5 medium supplemented with 0.2 mg/l NAA, 0.4 mg/l 2, 4-D and 0.2 mg/l BA (Figure 17). However, the media containing more concentration than 0.2 mg/l BA gave the compact callus from leave but the media supplemented with BA yielded the friable callus from the internode explants (Figure 18). The media containing 0.2 mg/l NAA, 0.4 mg/l 2,4-D and 0.1-0.5 mg/l kinetin were suitable for friable callus induction in both explant types. These media can also be used for callus induction from the other types of explants, such as petiole, lateral bud and root (Figure 19) which yielded friable callus with the average DW of 0.03, 0.05 and 0.03 g, respectively.

In case of internode explants, the best medium for friable callus induction was MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 0.5 mg/l kinetin (Figure 17). Somehow, this medium induced root formation and compact callus in leaf explants, which are not suitable for cell suspension culture.

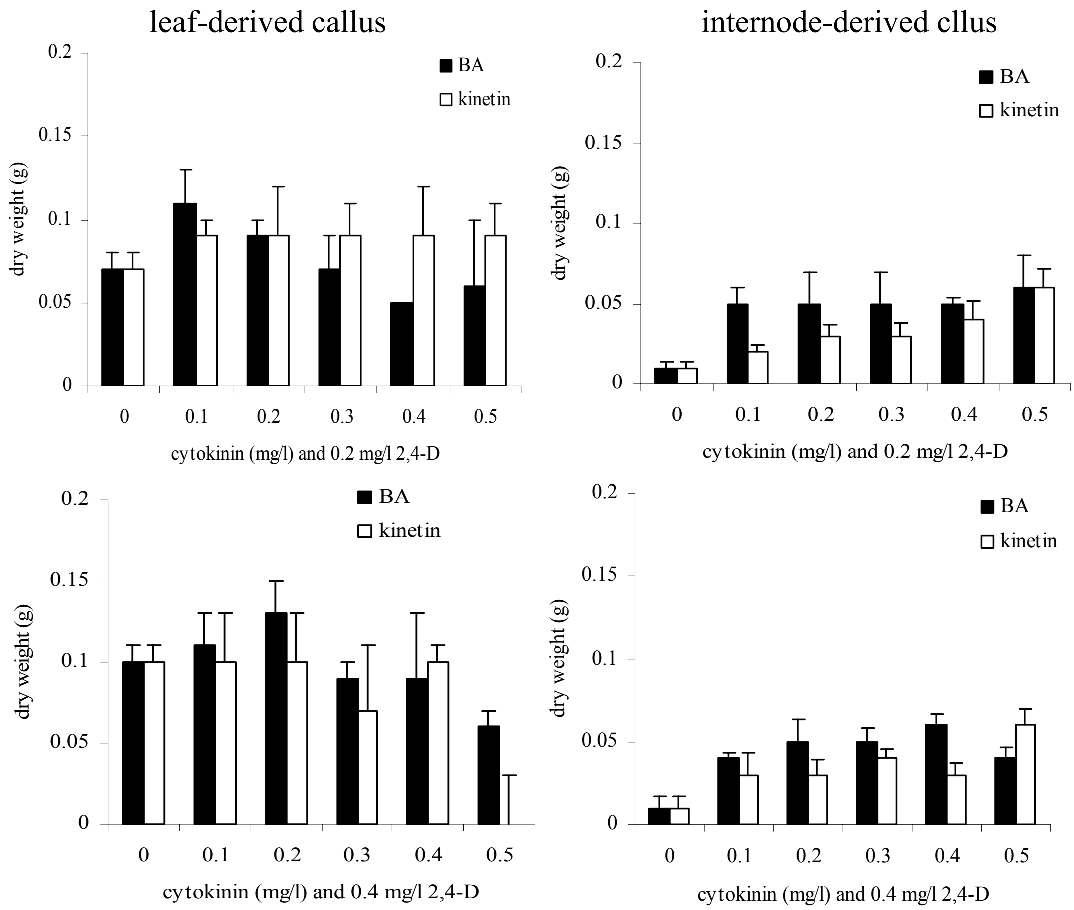

Figure 17 Effects of 2,4-D and cytokinin (BA and kinetin) on the growth of *Plumbago indica* L. leaf- and internode-derived callus. The basal medium was MS-B5. A constant concentration, 0.2 mg/l, NAA was added into each tested medium.

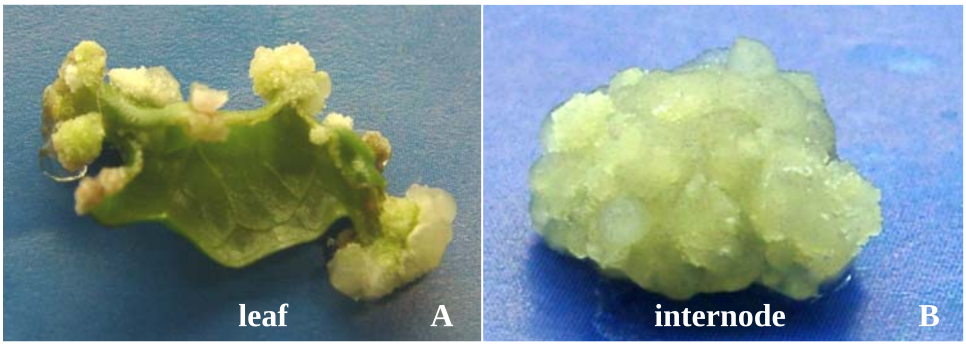

Figure 18 Compact callus (A) induced from leaf and friable callus (B) induced from internode of *Plumbago indica* L. on MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 0.2 mg/l BA.

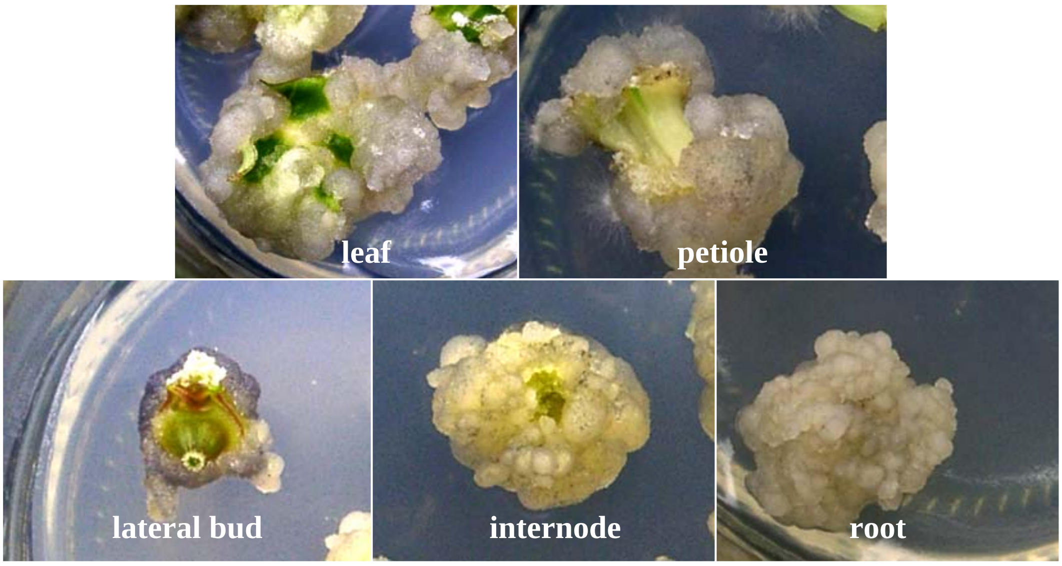

Figure 19 The friable calli induced from various explants of *Plumbago indica* L. cultured in MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 0.5 mg/l kinetin.

3.3 Effect of Auxin, Cytokinin and Culture Condition on Cell Suspension Cultures

3.3.1 Effects of 2,4-D, Kinetin and Culture Conditions on Growth of *P. indica* L. Cell Suspension Cultures

The preliminary experiments showed that friable callus was obtained from *in vitro* leaf, internode and root explant cultured in MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2 mg/l 2, 4-D and 0.5 mg/l kinetin. The calli were then transferred into liquid media comprised of the same constituents for cell suspension culture. The cultures were maintained in both dark and light (55µmol/m2/s 16h/d) conditions at 25±2 ^{0}C. The pattern of *P. indica* L. callus growing in liquid media was similar in both light and dark conditions in all explants tested. The pattern of suspension culture growth could be divided into two groups; the aggregated cells and the suspension cells. The slightly different in color of the culture was observed. In the light condition, the dark gray cell cultures obtained while the paler one obtained from dark condition (Figure 20).

For the growth study, the DGI of aggregated cells was not different among light condition and explants tested. In contrast, for the suspension cell, root explant showed higher DGI than the other explants in both light and dark conditions. However, light condition yielded higher DGI than the dark one (Figure 21).

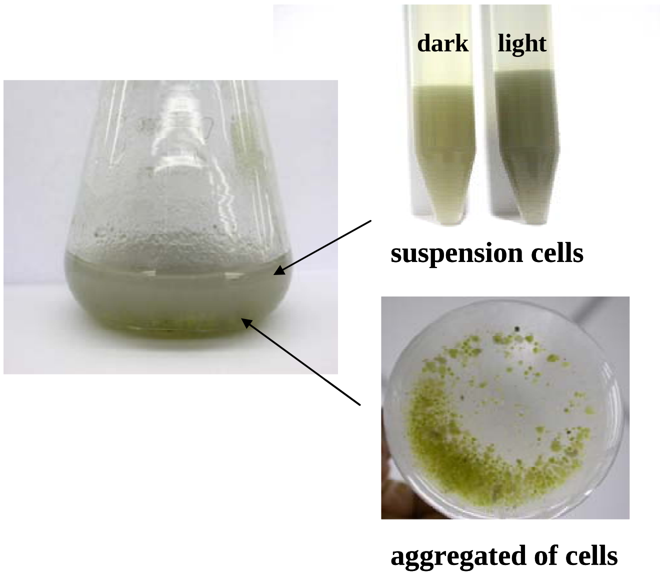

Figure 20 The growth patterns of *Plumbago indica* L. cell suspension cultures in MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 0.5 mg/l kinetin

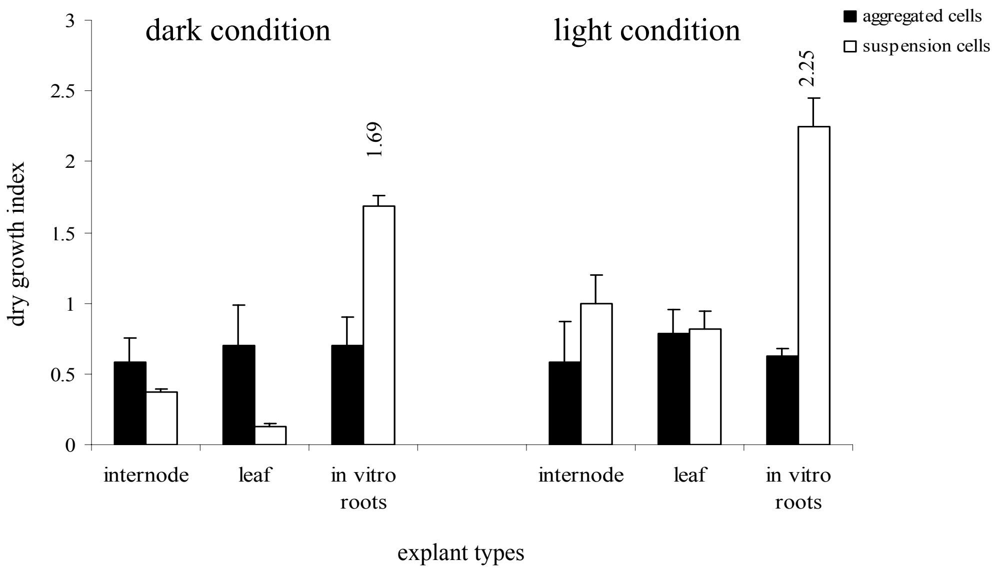

Figure 21 The DGI of cell suspension culture obtained from various explant parts in dark and light conditions. The basal medium was MS-B5. A constant concentration of 0.2 mg/l, NAA was added into each tested medium.

3.3.2 Effects of 2,4-D and Kinetin on Cell Growth

Due to the very low content of plumbagin in aggregated cells (preliminary result, data not shown), only suspension cells were studied in this experiment. The root-derived cell cultures were induced in MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2 mg/l 2, 4-D and 0.5 mg/l kinetin. They were shaken at 100 rpm on rotary shake in dark condition. The suspension cells were transferred into the same liquid media at every 2 weeks subculturing until homogenous cells obtained.

One g of 21-day-old homogenous cell culture was inoculated into MS-B5 medium supplemented with various concentrations of kinetin (0-0.5 mg/l) in combination with 2,4-D (0.2-0.6 mg/l) and a constant amount of NAA (0.2 mg/l). The growth of cell suspension cultures was studied in cell cultures at 21 days after subcultured. The increased of kinetin concentration tend to support the rapid cell growth, whereas 2,4-D at high concentration inhibited the cell growth. The culture media supplemented with 0.2 mg/l 2,4-D and 0.5 mg/l kinetin and a constant amount of NAA (0.2 mg/l) was the best medium for cell suspension cultures which yielded highest DGI of 4.24 as shown in Figure 22.

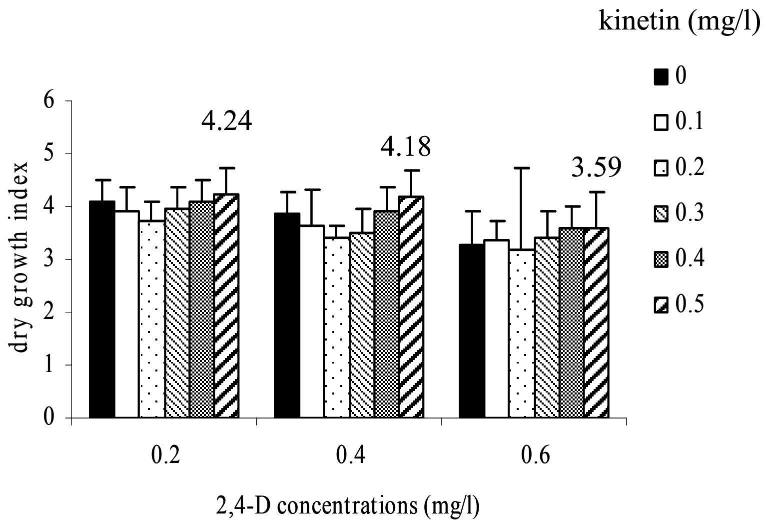

Figure 22 The DGI of 21 day-old, root derived *Plumbago indica* L. cell suspension cultures grown in dark condition in MA-B5 medium supplemented with a constant concentration of NAA (0.2 mg/l) and various concentration of kinetin.

4. The Production of Plumbagin in Various Types of Culture

This study represented the base line study of plumbagin production from various types of explants. The results of this study will help to plan the elicitation experiments in the next section. Three types of cultures, hairy root, callus and cell suspension were used and the results were described below.

4.1 The Production of Plumbagin in Hairy Root Culture

The studies on the effects of culture media strengths and sucrose concentrations on plumbagin production in hairy root cultures demonstrated that the lower strengths of culture media such as 1/2 B5, 1/4 MS and 1/2 MS were favorable for plumbagin accumulation in hairy root (Figure 23). Hairy roots cultured in 1/2 MS added with 20 g/l sucrose yielded the highest plumbagin content of 78.71 mg/l. Hairy roots cultures in 1/2 B5 medium plus 20 g/l sucrose also yielded high plumbagin content of 69.67 mg/l. In general, the cultures in MS medium showed the better growth of hairy root than the culture in B5 medium. The higher ratio of nitrate to ammonium in MS medium than in B5 medium may responsible for better growth of root (Jacob and Malpathak, 2005).

Beside the effect on plant growth, sucrose is also known to affect yield of secondary products (Jacob and Malpathak, 2004). In this study, culture media without sucrose proofed that they were not suitable for plumbagin production from hairy roots since they yielded only trace amount of plumbagin. Sucrose concentration of 20 g/l was the best concentration for plumbagin production in most media tested (Figure 23). At 20 mg/l sucrose, the maximum plumbagin production of 78.71 mg/l was obtained from 1/2 MS medium followed by 1/2 B5 and 1/4 MS media with the yield of 69.67 and 4.87 mg/l, respectively. Acording to the growth studies in the previous section (Figure 15), the DGI of hairy root in these media was slightly different. Thus, these 3 media will be selected for the elicitation study in the next section.

4.2 The Production of Plumbagin in Callus Culture

The plumbagin production from the 4-week-old callus cultured on 22 different nutrient media was analyzed using HPLC. Result indicated that the accumulation of plumbagin in *P. indica* L. callus can be effectively modified with auxin and cytokinin supplemented into culture media (Figure 24). In this experiment, the increasing of 2,4-D concentration from 0.2 mg/l to 0.4 mg/l was led to the decrease of plumbagin production in both leaf- and internode-derived callus. The plant growth regulators concentrations were reported to be crucial factors in secondary metabolites production. The type and concentration of auxin, cytokinin or auxin/cytokinin ratio alter dramatically both the growth and compound produced in plant cell culture. The 2,4-D has been shown to inhibit the production of secondary metabolites in a large number of plants. Elimination of 2,4-D or replacement of 2,4-D by NAA or indole acetic acid (IAA) has been shown to enhance the production of anthocyanins in callus cultures of *Populus sp.* and *Daucus carota* (Rajendran *et al*., 1992).

Plumbagin content in calli obtained from leaf and internode explants showed significant different when cultured in the media contained two different levels of 2,4-D and the six different levels of kinetin or BA were supplemented. In case of using kinetin, the results illustrated the inconsistency of plumbagin production but calli were more friable than those in the BA containing media. Considering the sources of explant, the leaf explant provided the higher plumbagin content than that of internode explant. However, the plumbagin content in calli was much lower when compared to the content in hairy root. In this case, calli may not be of interest for elicitor studies.

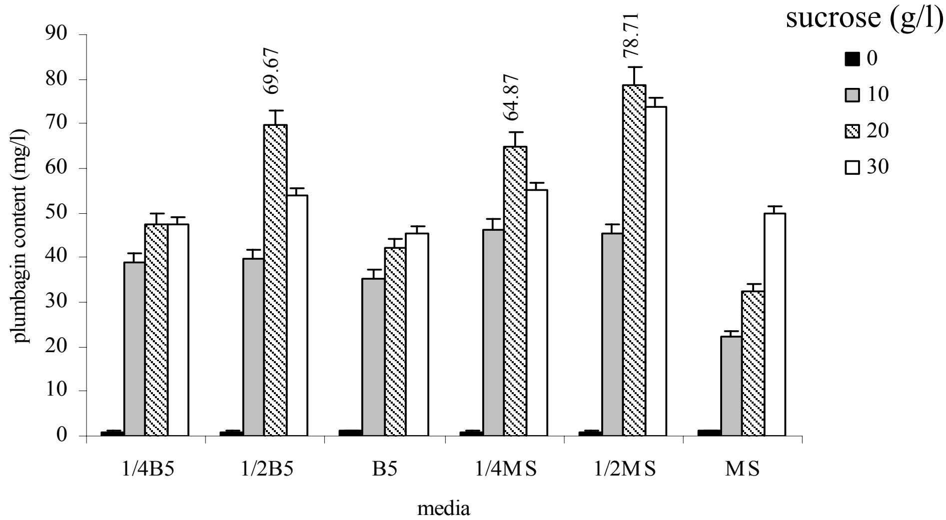

Figure 23 Effects of culture media and sucrose concentrations on plumbagin production of *Plumbago indica* L. hairy root culture

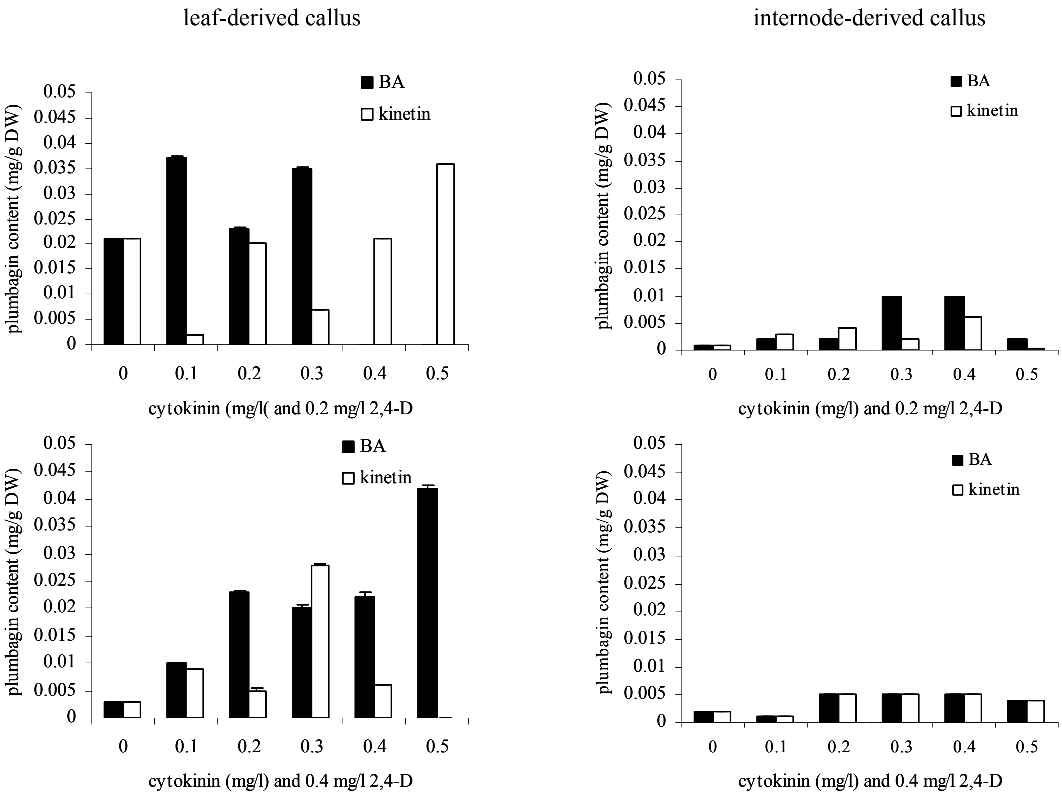

Figure 24 Effects of cytokinin (BA and kinetin) and 2,4-D on plumbagin content in leaf- and internode-derived callus of *Plumbago indica* L.

4.3 The Production of Plumbagin in Cell Suspension Culture

4.3.1 The effects of plant growth regulators, light and cell type on plumbagin production

The effects of plant growth regulators and light conditions on plumbagin production were examined using cell culture obtained from internode-derived callus and leaf-derived callus. Suspension cells were initiated in the medium of the best result from the culture establishment section which was MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2 2,4-D and 0.5 mg/l kinetin. As cell growth pattern could be separated into 2 types as described in section 3.3.1. The plumbagin content was also determined in both cell types in all treatments. Moreover, with this type of culture, the secretion of plumbagin into the media was also examined. The results demonstrated that the plumbagin production was correlated to the cell types. The aggregated cell accumulated only trace amount of plumbagin. Similar results were reported by Bolta *et al*. (2000) in cell suspension culture of *Salvia officinalis* which the compact cell types yielded lower ursolic acid than in the friable cells. Furthermore, the aggregate cells with the size of less than 4 mm found to accumulate secondary metabolites as reported for jaceosidin production in cell cultures of *Saussurea medusa* (Zhao *et al*., 2003) and isocampotothecin A and B production in cell culture of *Camptotheca acuminate* (Yu *et al*., 2005).

The cell aggregates can be few millimeters in diameter and contain hundreds of cells. Different plant species differ in the size distribution of the aggregates. The aggregates size distribution also changes with the phase of growth. Because metabolic production is not always the function of a single cell but involves different cells, plant cell aggregation may be a favorable property with respect to the production of secondary metabolites (Fuller, 1984).

Cultures incubated under light yielded lower plumbagin content than under dark condition. Similarly, internode-derived culture was also lower in plumbagin content than that of root-derived culture (Figure 25, 26 and 27). It seems that light affects some enzymatic pathways which lead to the inhibition of the biosynthesis of plumbagin. Most naphthoquinone are derived from shikimate pathway and the plumbagin is derived from six C_2 units (Durand and Zenk, 1971). The shikimic acid pathway is a major route for biosynthesis of aromatic compounds in plants and microorganisms including the proteinaceous amino acids such as phenylalanine, tyrosine and tryptophan. The shikimic acid pathway provides precursors for many ubiquitous compounds important in the life of the plant such as the structural element lignin, the plant growth hormones and quinones. The products of this pathway are also important in the responses of plant to environmental stimulants (McCue and Conn, 1990).

Light is an important factor for the accumulation of cell biomass and formation of secondary metabolites. The stimulatory effects of light on the formation of compounds have been shown including flavonoid from cell cultures of *Petroselinum hortense* (Krewzaler, 1973) and anthocyanins from cell cultures of *Camptotheca acuminate* (Pasqua *et al*., 2005). On the other hand, light has an inhibitory effect on the accumulation of secondary compounds such as shikonin, a red naphthoquinone from hairy root and cell suspension cultures of *Lithospermum erythrorhizon* (Yazaki *et al*., 2001). Cell cultures of *Hypericum perforatum* L. incubated in darkness showed an increase in growth and hypericin production (Walker *et al*., 2002).

The 2,4-D at high concentrations inhibited plumbagin production and the content accumulated in cells. The 2,4-D has been shown to inhibit the production of secondary metabolites in a large number of cases. The elimination of 2,4-D or concentration of its replacement by NAA or IAA enhance anthocyanin, betacyanins, shikonin and anthraquinone (Roa and Ravishankar, 2002). Oppositely, high kinetin was effective in promoting plumbagin content (Figure 25 and 26). Kinetin stimulated the anthocyanin production in *Haplopapus gracilus* but inhibited the formation of this secondary metabolite in *Populus* cell cultures (Roa and Ravishankar, 2002). The highest plumbagin content of 13.66 mg/l was obtained from root- derived cell; 0.019 mg/l from aggregated cell, 9.58 mg/l from suspension cell and 4.06 mg/l from culture medium, cultured in MS liquid medium supplemented with 0.2mg/l 2,4-D, 0.5 mg/l kinetin with a constant amount of 0.2 mg/l NAA incubated under dark condition (Figure 26). Then this medium was chosen as the medium to further elicitation study.

4.3.2 Relationship between Cell Growth and Plumbagin Production

An important factor for enhancing the plumbagin production was the stage of cell growth for elicitor treatment. Cell cultures generally have extended cell cycle times due to longer G1 phase for the synthesis of enzymes necessary for DNA replication. Then, cell at different stage of growth cycle with different level of mRNA and protein may yield varied responses in term of cell growth and secondary metabolites (Chong *et al*., 2005). It is believed that the stronger stimulation of secondary metabolites by biotic elicitor usually occurred in the late exponential growth stage of plant cell culture (Buitelar *et al*., 1992). However, the polyacetylene production in *Ambrosia maritima* was challenged with elicitor during the early stage of growth. The high availability of the nutrient in the medium or cells can be used for secondary metabolites biosynthesis (Zid and Orihara, 2005). There is no rule as to the best time in the culture cycle to harvest cells in order to obtain maximum yield of the secondary metabolites. Thus, it is best to produce large amounts of biomass under rappid growth conditions and then transfer the accumulated biomass to the second stage of culture to promote secondary metabolite production (Chawla, 2000).

Since growth and plumbagin production are strongly affected by culture media, light conditions, explants parts, the liquid MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 0.5 mg/l kinetin was selected for this experiment. The selected explant was root-derived suspension cell as it was shown to be the suitable explant as described earlier. The experiment was carried out in dark condition. The duration time of experiment will help to determine the right age of culture in the latter elicitation experiment.

The characterization of growth curve of *P. indica* L. cell cultures revealed 3 days of lag phase, followed by an exponential phase lasting until day 24 of culture and then stationary phase (Figure 28). The period from day 6 to day 24 was the exponential phase. The cells grew fast and the biomass reached the highest DGI of 5.62 on day 24. At the last phase of growth curve, cell death occurred resulting in browning of culture.

Plumbagin productions both in cells and culture media were highly correrelated to cell growth during the culture period of first 12 days especially in the amount released into culture medium. The amount of plumbagin accumulated in cells was, actually, constant throughout the whole period of 27 days (Figure 28). Interestingly, after 12 days, plumbagin released into culture medium decreased rapidly as cells reached the mid-exponential phase. This result indicated that during middle phase of culture, cells were in rapid division. As nutrients in the medium became more limiting, cell divisions decreased. The synthesis of secondary product appeared to be stimulated if fixed carbon were not fully utilized by the primary metabolic activities of cell growth and differentiation and converted into secondary compounds. This rapid growth is resumed in cell suspension culture after the secondary products are degraded (Collin and Edwards, 1998; Chawla, 2000). The *P. indica* L. cells changed from pale gray to brown after 21 days. This is suggested that the browning coloration can not be direct linked to the induction of plumbagin, but rather could be due to the other phenolic compounds induced from stress. It may also suggest the onset of cell necrosis or possibility induction of apoptosis mechanism (Yuan *et al*., 2002). Incaseof *P. indica* L., cell suspension must be subcultured within the 3 weeks, otherwise the culture including the secondary metabolites of interest will be lost.

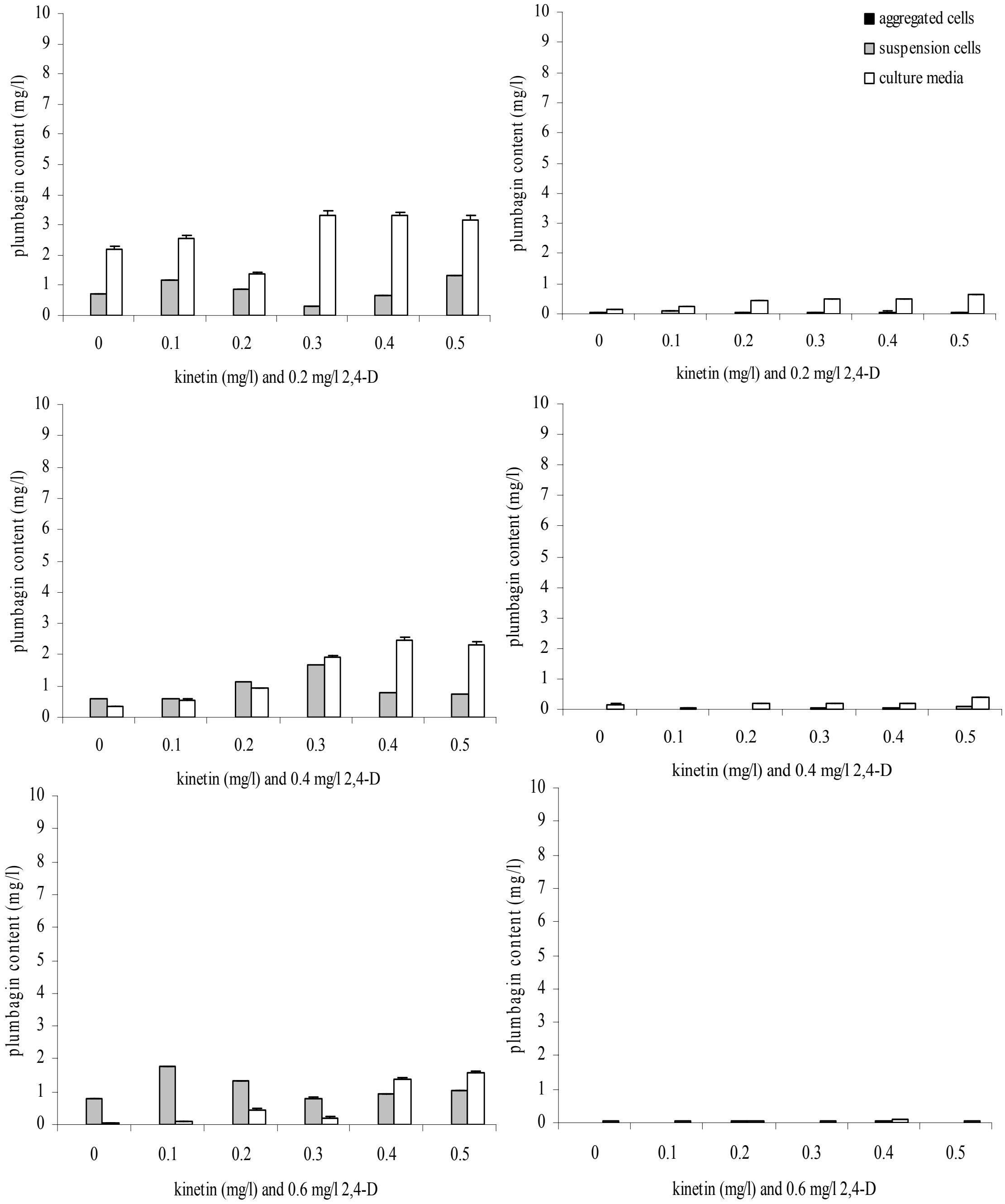

Figure 25 Effects of kinetin and 2,4-D concentrations on plumbagin content of *Plumbago indica* L. internode-derived cell culture under dark and light conditions. The basal media was MS-B5 and a constant concentration of 0.2 mg/l NAA was added into each tested medium.

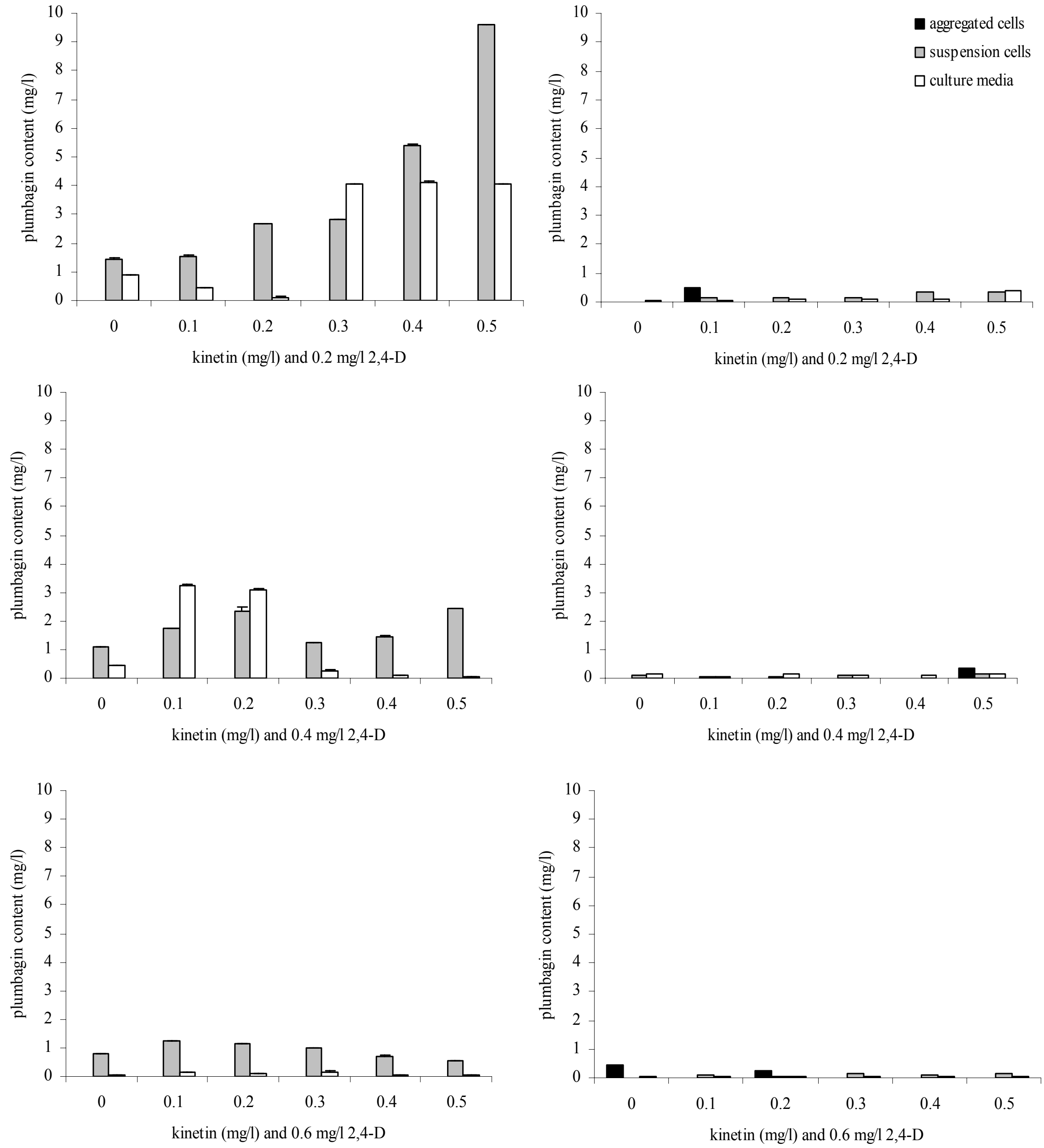

Figure 26 Effects of kinetin and 2,4-D concentrations on plumbagin content of *Plumbago indica* L. root-derived cell culture under dark and light conditions. The basal media was MS-B5 and a constant concentration of 0.2 mg/l NAA was added into each tested medium.

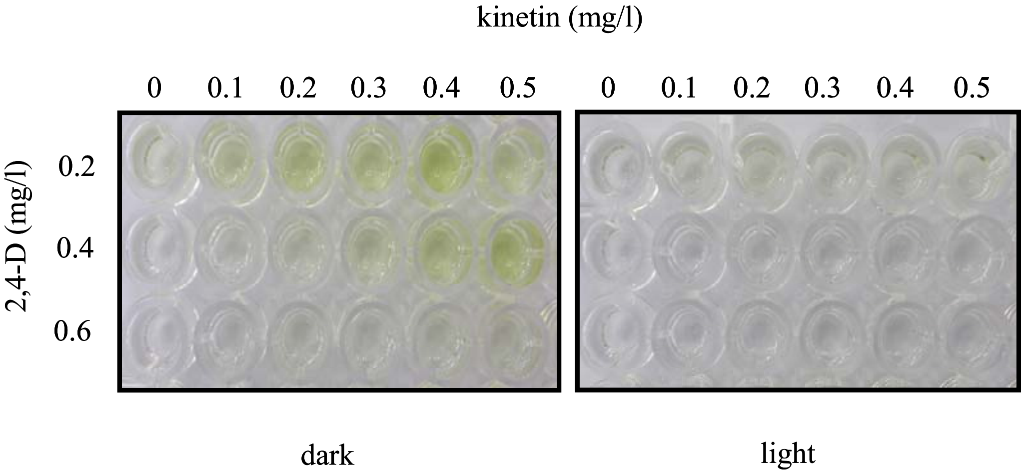

Figure 27 Plumbagin released from root-derived cell cultures into the culture media (MS-B5 medium supplemented with 0.2 mg/l NAA, 0.2-0.6 mg/l 2,4-D and 0-0.5 mg/l kinetin) after harvested cell.

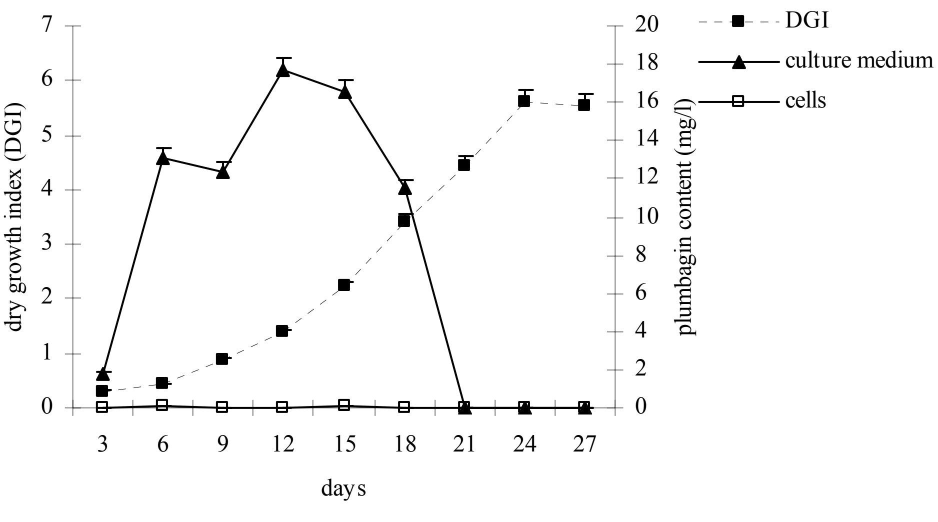

Figure 28 Growth curve of *Plumbago indica* L. cell suspension culture and plumbagin accumulation in cells and in culture medium.

5. Effects of Biotic and Abiotic Elicitation of Plumbagin in Hairy Root and Cell Suspension Cultures

During elicitation process, plant cell recognizes the elicitor by molecular interaction between plant receptor at the cell membrane surface or cytoplasm and low molecular signal legends from fungal cells. The signal perception by plant cells is followed by the elicitor signal transduction and the occurrence of plant defence responses, such as defence genes activation and some defence mechanisms related secondary metabolite accumulation (G´omez, 2004; Zhao *et al*. 2001).

Chitin, a linear polysaccharide composed of (1→4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc) residues, and chitosan, the fully or partially de-N-acetylated derivative of chitin composed of (1→4)-linked GlcNAc and 2-amino-2- deoxy-β-D-glucopyranose (GlcN)), have been proposed as elicitors of defense reactions in higher plants (Vander *et al.,* 1998; Ortmanne *et al*., 2004). Chitosan elicitor is one of the most commonly used elicitor for the induction or enhancement of secondary metabolites in cell cultures of several medicinal species (Kim *et al*., 1997; Jin *et al*., 1999; Luo and He, 2004; Al-Gendy and Lockwood, 2005) There also have been reports on the production of diosgenin in hairy root of *Trigonella foenum-graecum* L. (Merkli *et a*l., 1997) and the production and release of tropane alkaloid in transform root of *Brugmansia candida* (Pitta-Alvarez and Guilietti, 1999). There have been reported the use of chitin or chitosan elicitor for enhanced production of plumbagin in cell suspension cultures of *Drosophyllum lusitanicum* (Nahálka *et al*., 1998) and plumbagin from cell suspension cultures of *Plumbago indica* (Komaraiah *et al*., 2002; Komaraiah *et al*., 2003).

Hairy root and cell suspension cultures of *P. indica* L. were elicited with elicitors and the effects on plumbagin accumulation were studied. Dosage responses were performed to determine the effects of elicitor concentrations on growth and plumbagin production. One concentration of effective elicitors from the dosage studies was chosen for exposure times. Cultures were harvested and analyzed for intracellular and extracellular plumbagin accumulation at the different time after elicitation. Secretion of plumbagin into the medium was also detected in both hairy root and cell suspension cultures in response to elicitation.

5.1 Effects of Elicitors Concentration on Hairy Root Culture

5.1.1 Elicitation with Chitin

Hairy roots were cultured in 3 selected liquid media; 1/4 MS, 1/2 MS and 1/2 B5 for 21 days and then treated with chitin at various concentrations. Cultures were harvested on 8 days after elicitation. The dry growth index (DGI) was calculated for the elicited and control cultures. Hairy roots treated with chitin decreased biomass in all media tested as compared with unelicited ones (control). The elicited hairy roots shown less growth in lower salts of medium strength such as 1/4 MS, but their biomass were increased slightly in liquid medium supplemented with 100 mg/l chitin. The DGI of the elicited root grown in this medium decreased from 2.7 to 1.95 (Figure 29).

Chitin at various concentrations did not enhance the plumbagin accumulation in hairy roots in all media tested. The plumbagin content in elicited hairy roots was lower than that unelicited hairy roots (control), it may due to the reduction of the hairy roots growth rate. It was observed that plumbagin was slightly released into the medium during culture shaking for 8 days after elicitation and then were harvested. Plumbagin released into the culture medium was observed between harvesting the cultures by stopped shaking at least 6 h and continuous shaking before separated the hairy roots from the liquid media. The results showed that the cultures which stopped shaking at least 6 h increased plumbagin releasing into the liquid media (Figure 30). The hairy roots elicited with chitin and cultured in 1/4 MS or 1/2 B5 liquid medium were in favor of releasing the plumbagin into the media. However, the plumbagin releasing was found in the control hairy root cultured in 1/2 B5 medium. This result indicated that low salts of culture medium and stopped the shaking were possible the cause of hairy roots stress and rapid secretion the plumbagin into the medium. The high plumbagin released into all culture media was obtained from hairy root treated with 500 mg/l chitin (Figure 29).

This experiment demonstrated that different culture media had effects on growth and plumbagin production in hairy root culture after elicited with chitin especially low salts of medium strength had greatly affected the release of plumbagin into the culture medium. Hairy root cultures of *P. indica* L. in half-MS medium showed increase growth while half-B5 medium showed maximum secondary metabolite production. Gamborg's B5 vitamins differ from the vitamins of MS in having a high concentration of thiamine. Thiamine is involved in cell biosynthesis and metabolism (Willims, 1995). The plumbagin releasing also depends on culture stress techniques such as stopped shaking before hairy roots were harvested. Addition of chitin in hairy root culture resulted in increased biosynthesis of plumbagin and it was released into the 1/4 MS liquid media which approximately 3 folds compared to the control but hairy root yielded low DGI. However, elicitation of plumbagin with chitin in hairy root cultures may be depend on various effects such as concentration of elicitor, time to contact with elicitor and growth stage of the culture (Bhagwath and Hjortsø, 2000). There has been reported that the stronger stimulation of secondary metabolites by biotic elicitor usually occurred in the late exponential growth stage of culture (Buitelar *et al*., 1992).

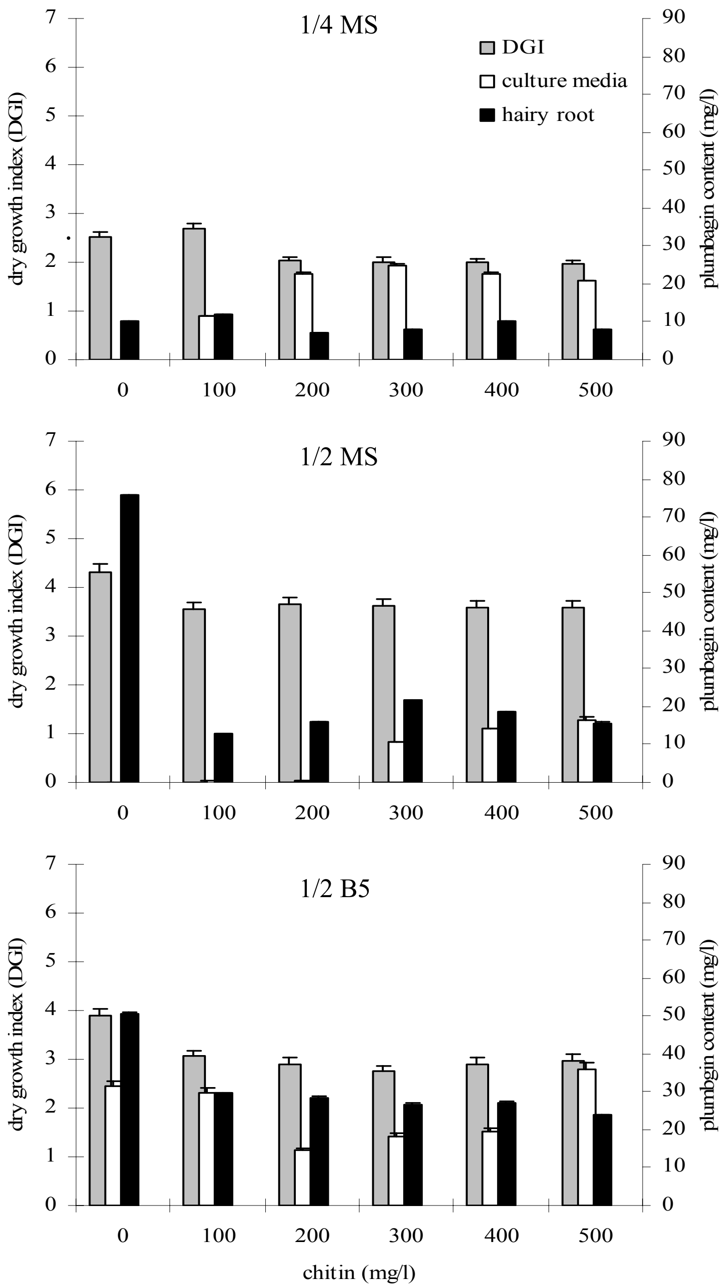

Figure 29 Effect of chitin at different concentrations on growth, the accumulation and release of plumbagin in hairy root cultures of *Plumbago indica* L. on day 8th after elicitation.

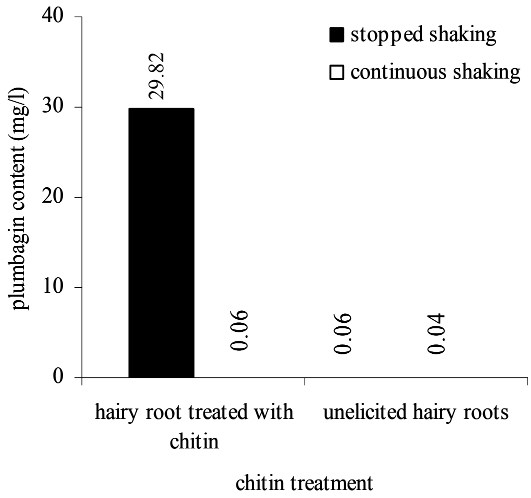

Figure 30 Plumbagin contents in culture liquid medium as compared between stop and continuous shaking.

5.1.2 Elicitation with Chitosan

a) Effects of Chitosan and Culture Media on Growth and Plumbagin Production

In this experiment, hairy roots were also cultured in 3 selected liquid media as described in 5.1.1 and were harvested on day 5th after elicitation. Chitosan addition had slightly affected on hairy roots growth in MS basal strength liquid media. The DGI of hairy roots was decreased when higher concentrations of chitosan 300-500 mg/l were used.

Chitosan addition at various concentrations had effects on both plumbagin accumulation in hairy roots and the released of plumbagin into the media tested. Hairy roots grown in lower salts of medium strength such as 1/4 MS and elicited with chitosan were favorable for releasing the plumbagin into the medium. The hairy roots treated with high concentrations of chitosan (300-500 mg/l), the plumbagin accumulation in hairy roots was decreased in all media. In the other hand, 100-200 mg/l chitosan induced plumbagin accumulation in hairy roots compared with the untreated one (control). Chitosan treatments reflected positively on plumbagin releasing into the medium compare to the control, especially when the high concentrations of chitosan (300-500 mg/l) were used in case of half-B5. Chiosan led to a decrease in dry matter. In case of hairy root cultured in 1/4 MS and 1/2 MS medium treated with chitosan, the growth of hairy root was not different. The plumbagin was released into 1/4 MS medium more than that of 1/2 MS medium. This indicated that lower salt of medium may effect on plumbagin production. Secondary pathways are activated in response to stress. The optimal concentrations of chitosan for releasing the plumbagin into all selected media were 400 or 500 mg/l on day 5th after treated with chitosan (Figure 31). Addition of chitosan in hairy root culture resulted in increased plumbagin production which approximately 3-4 folds compared to the control (H_2O).

In order to increase the plumbagin content, hairy roots grown in half- MS liquid medium adding with chitosan at high concentrations (500-2,500 mg/l) were also studied and 0.1 % acetic acid was used as control. The DGI of hairy roots was decreased when increased the chiotosan concentrations. The plumbagin was excreted into the culture medium on day 2nd after elicitation with chitosan. High concentration of chitosan stimulated the plumbagin released into the media faster than the low concentrations of chitosan. However, addition of chitosan at high concentrations in hairy root culture was not resulted in increased plumbagin production higher than chitosan at low concentrations, approximately 2-3 folds compared to adding 0.1 % acetic acid (control) (Figure 32) and 3-4 folds compared to adding water (Figure 31). The plumbagin releasing into liquid media was the effect of combination between chitosan and acetic acid. This would suggest the involvement of acetic acid in this response by breaking chitosan into units that are more active in promoting the release of plumbagin. Similar has been reported that highest concentration of chitosan (1000 mg/l) with 1% acetic acid (pH 5.5) induce the released of hyoscyamine after 24 and 48 h and scopolamine throughout the 72 h of exposure from hairy root culture of *Brugmansia candida* treated with (Pitta-Alvarez and Giulietti, 1999).

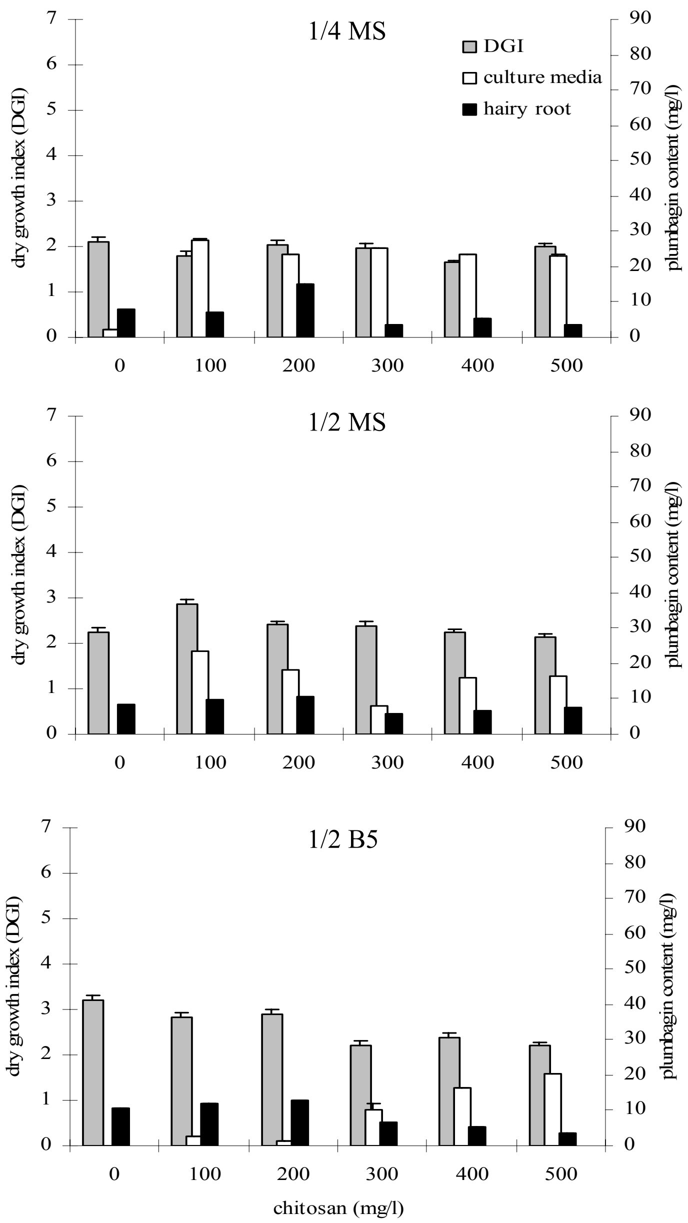

Figure 31 Effect of chitosan at different concentrations on growth, and the accumulation and release of plumbagin in hairy root cultures of *Plumbago indica* L. on day 5th after elicitation.

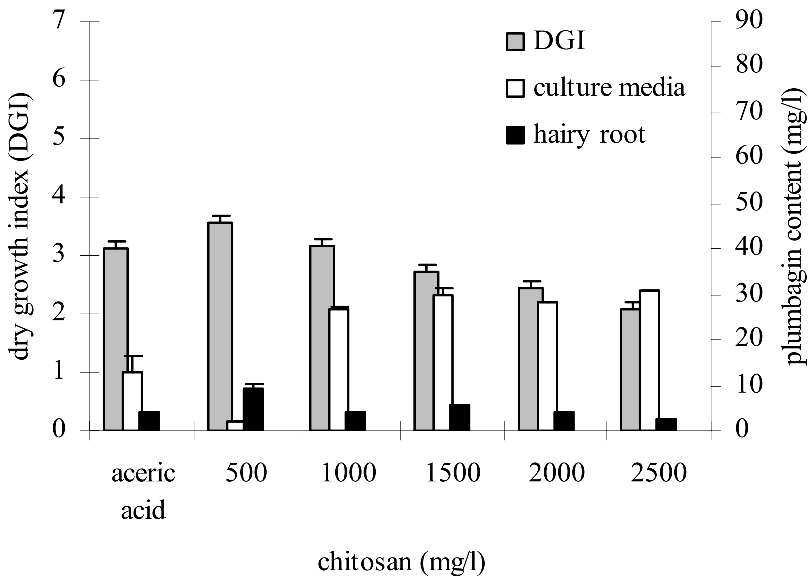

Figure 32 Effect of chitosan at different concentrations on growth, and the accumulation and release of plumbagin in hairy root cultures of *Plumbago indica* L. in 1/2 MS liquid medium on day 2^{nd} after elicitation.

b) Effects of Acetic Acid on Growth and Plumbagin Production

There have been reports that low pH medium had effected the production and release of tropane alkaloids in roots of *Datura stramonium* (Sáenz-Carbonell *et al*., 1993). This positive effect of low pH was found in case of using acetic acid as a solvent for preparation of chitosan. For example, acetic acid and chitosan stimulated the synthesis of scopolamine and hyoscymine and promoted release of both alkaloids in root of *Brugmansia candida* (Pitta-Alvarez and Guilietti, 1999). Since chitosan preparation solution stock was dissolved in acetic acid 2% v/v and diluted at concentration between 100-500 mg/l chitosan. Thus, in this experiment, acetic acid was tested at calculated concentrations (0-0.1%) depend on chitosan solution at the concentrations of 0-500 mg/l in order to confirm that acetic acid may effect on stimulation the plumbagin production. 1/2 MS liquid media was used and all concentrations of acetic acid adding were adjusted at pH 5.7.

Acetic acid had positive effect on growth of hairy roots. The cultures were harvested on day 5th after elicitation similar to the experiment of hairy roots treated with chitosan. The DGI of the elicited hairy roots cultures was lower than that of the control. High concentrations of acetic acid, especially more than 0.04% v/v, the DGI of hairy roots was decreased (Figure 33).

The plumbagin accumulation in the hairy roots after elicited with acetic acid was decreased when the concentrations of acetic acid were increased. The slightly release of plumbagin into the liquid media was observed in acetic acid treatment at the concentration of 0.06-0.1 % v/v on day 5th of exposure. This results demonstrated that acetic acid (medium = pH 5.7) was slightly effect on stimulation the plumbagin released into culture medium (Figure 33). Then, the used of chitosan in combination with acetic acid, the majority effect on the release of plumbagin into the media was chitosan. In some hairy root culture, acetic acid stimulated the release of secondary metabolites such as scopolamine and hyoscyamine by acting as stress inducing agent (Pitta-Alvarez and Guilietti, 1999).

The optimized use of chitin, chitosan (in its combination with acetic acid) and acetic acid could be advantage for large scale production of plumbagin. Acetic acid could be breaking down chitosan into units that are more active in promoting the released of secondary metabolites (Pitta-Alvarez and Giulietti, 1999). When the cultures were treated with these elicitors, the plumbagin was released into the 1/2 MS liquid culture medium while the unelicited hairy roots was not released this compound as shown in Figure 34

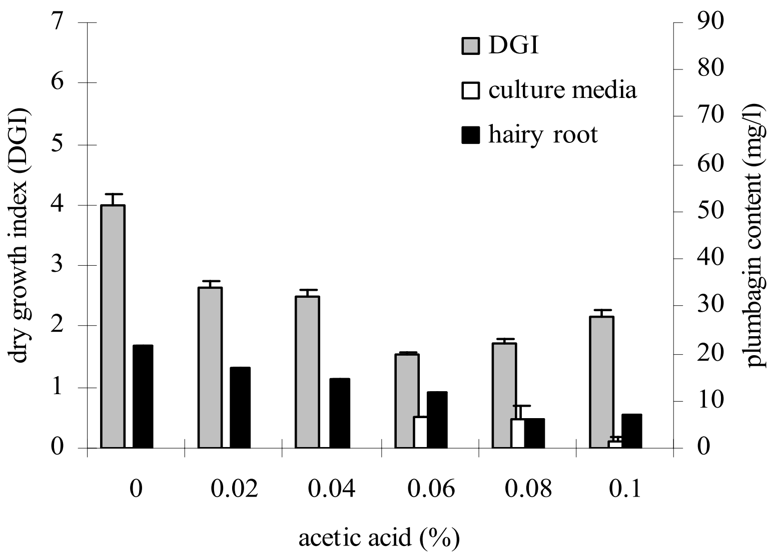

Figure 33 Effects of acetic acid at different concentrations on growth and the accumulation and release of plumbagin in hairy root cultures of *Plumbago indica* L. in 1/2 MS liquid medium on day 5th after elicitation.

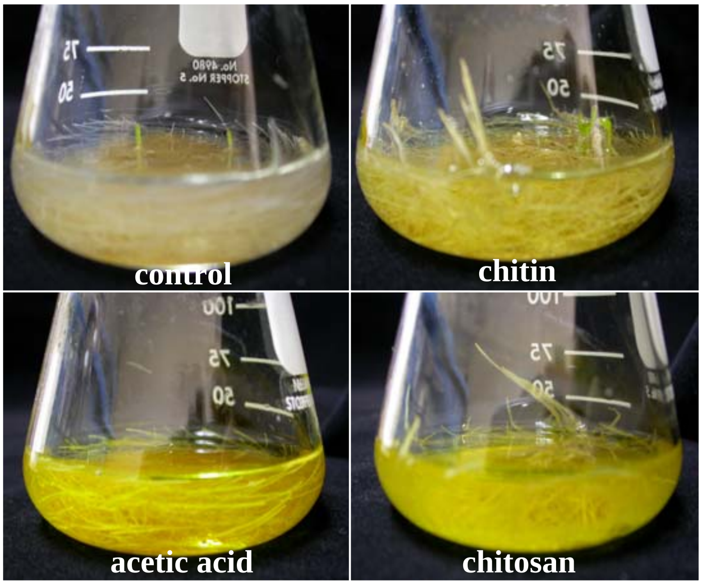

Figure 34 The plumbagin was released into 1/2 MS liquid culture medium after elicited with various elicitors compared to control as indicated by the color of the medium.

5.1.3 Other Elicitors

a) Effects of Yeast Extract on Growth and Plumbagin Production

The growth responses of *P. indica* L. hairy roots to elicit with the yeast extract elicitor are shown in Figure 35. The yeast extract elicitor treatments were less effective on hairy roots growth compared to the control. However, yeast extract at high concentrations tended to improve the hairy roots growth because yeast extract is composed of a variety of compounds, apart from amino acids, vitamins, and minerals. It is also possible that elicitation effects might be due to another component still not identified (Ertola and Hours, 1998).

Yeast extract is one of the most commonly used elicitors for the induction or enhancement of secondary metabolites production such as tanshinone production in hairy roots of *Salvia miltiorrhiza* (Chen *et. al*., 2001; Ge and Wu, 2005), alkaloid production in hairy roots of *Brugmansia candida* (Pitta-Alvarez *et al*., 2000). In this experiment, the plumbagin content was determined and compared untreated with the yeast elicitor-treated hairy root cultures including it released into liquid media. Yeast elicitor had effect on plumbagin accumulation only in hairy root, but not on releasing of the plumbagin into the culture media. The plumbagin content in hairy roots was decreased when increased the concentrations of yeast elicitor. The maximum plumbagin content in hairy roots (41.70 mg/l) was 2.39-fold by 50 mg/l yeast elicitor over the control (17.39 mg/l).

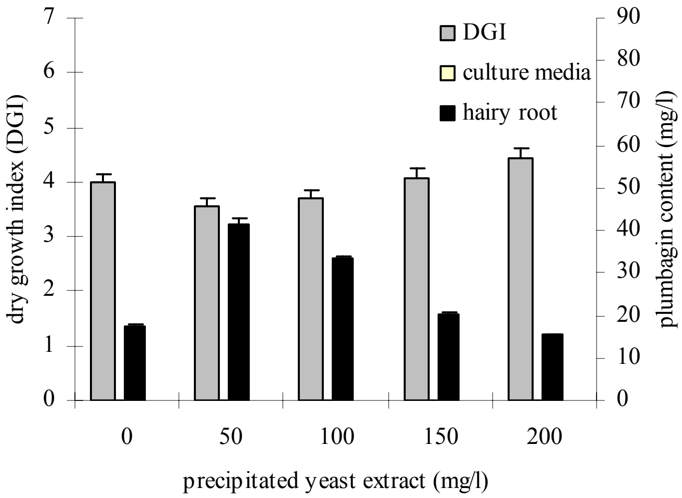

Figure 35 Effect of precipitated yeast extract at different concentrations on growth, and the accumulation and release of plumbagin in hairy root cultures of *Plumbago indica* L. on day 8th after elicitation.

b) Effects of Fungal Elicitor on Growth and Plumbagin Production

The growth responses of *P. indica* L. hairy roots elicited with the fungal elicitor obtained from homogenate mycelium of *Colletotrichum capsici* was shown in Figure 36. The fungal elicitor treatments were less effective on hairy roots growth compared to the control.

Fungal elicitor had not effect on plumbagin accumulation in hairy root and the release of plumbagin into the culture media. There was no difference in the response with the concentrations of fungal elicitor. However, the fungal elicitor at all concentrations inhibited plumbagin production.

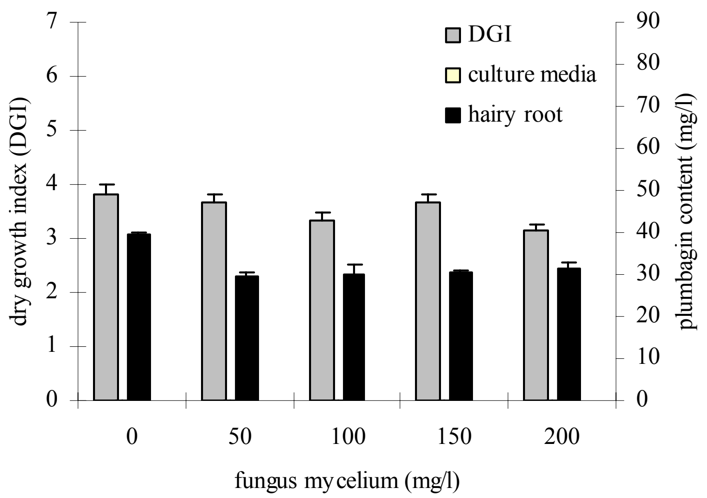

Figure 36 Effects of fungal mycelium at different concentrations on growth, the accumulation and release of plumbagin in hairy root cultures of *Plumbago indica* L. for 7 days of elicitation.

5.2 Time of Exposure to Chitin and Chitosan Elicitors in Hairy Root Culture

For determination of the effect of elicitation changing period on plumbagin production by hairy root culture of *P. indica* L. was conducted. Five hundred mg/l chitin were added to the same liquid medium and the plumbagin content were recorded on 1-4th day after elicitation. Four hundred mg/l chitosan were also added to the 1/2MS and 1/2B5 liquid medium and recorded on 1-7th day after elicitation.

The plumbagin in hairy roots treated with chitosan was released into 1/2MS and 1/2B5 liquid on 5 days while the plumbagin in hairy roots treated with chitin was slightly released into the medium on 8th day after elicitation. The maximum plumbagin content in hairy roots elicited with chitin was 75.05 mg/l (7.78 mg/l in 1/2 MS liquid medium and 67.23 mg/l in hairy roots). The maximum plumbagin content in hairy roots elicited with chitosan was 46.88 mg/l (37.36 mg/l in 1/2 MS liquid medium and 9.52 mg/l in hairy roots). This reveals that the duration of cultivation time after elicitation with chitosan rather important with respected to plumbagin production. The plumbagin was released into the culture medium after elicited with chitin was lower than that elicited with chitosan and take longer time to release into the media. This might have been due to solubility of chitin is more difficult than chitosan. However, chitin elicitor yielded plumbagin accumulation in hairy root was better than used chitosan as elicitor (Figure 37 and 38).

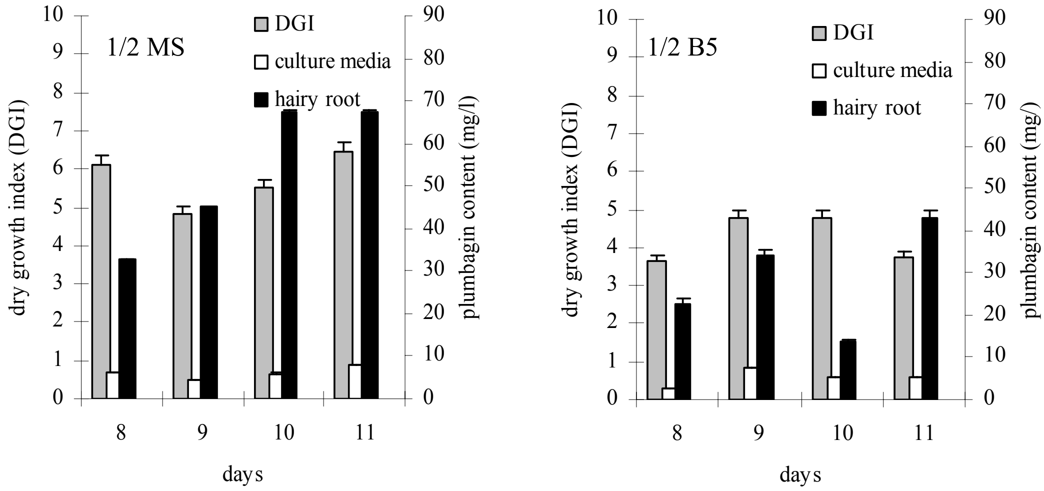

Figure 37 Growth and plumbagin content (mg/l) of hairy root culture harvested at different time of elicitation with 500 mg/l chitin.

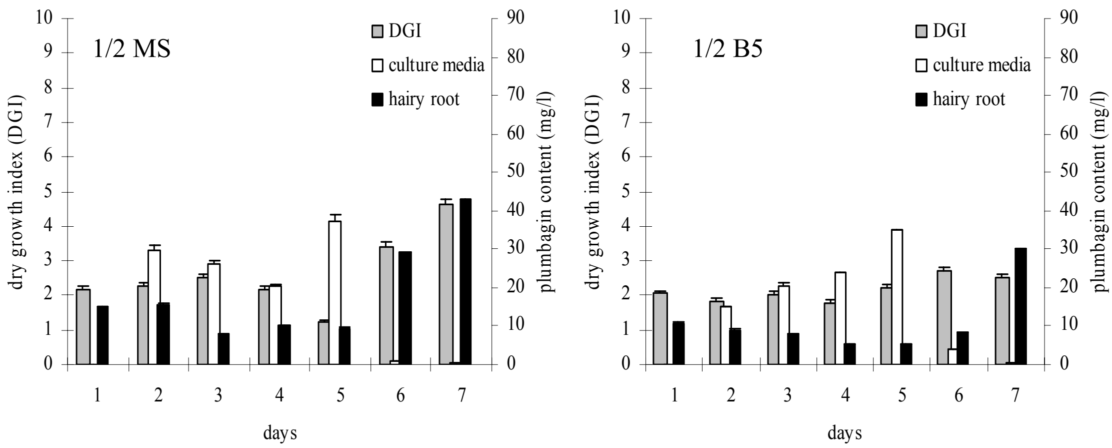

Figure 38 Growth and plumbagin content (mg/l) of hairy root culture harvested at different time of elicitation with 400 mg/l chitosan.

5.3 Effects of Elicitors on Cell Suspension Culture

5.3.1 Elicitation with Chitin

P. indica L. cell suspension cultures were elicited with chitin and cultures were harvested on day 3rd after elicitation. The DGI of cell fluctuated in the range of chitin concentrations (100-500 mg/l). However, the cell growth was lower than the control. The cell color changed from gray to yellow cells when the cultures were elicited with 300-500 mg/l chitin which could be seen on day 3rd after elicitation (Figure 39).

Figure 40 shows chitin added at various concentrations had less plumbagin content in the cells. Cultures treated with chitin at high concentrations tend to increase the plumbagin accumulation in the cells. Furthermore, chitin also stimulated the release of plumbagin on day 3rd after elicitation. The highest plumbagin content which released into the medium was found after elicited with 500 mg/l chitin (20.29 mg/l). Addition of chitin in cell culture was increased plumbagin production about 20 folds compared to the control. However, there had not found the crystallization of plumbagin in the cultured cells of *P. indica* L. whereas there has been reported in cell suspension culture of *Drosophyllum iusitanicum* (Nahálka, *et al*., 1998).

0 100 200 300 400 500

chitin (mg/l)

Figure 39 The change of cell color from gray to yellow after chitin elicitation at different concentrations in *Plumbago indica* L. cell suspension culture.

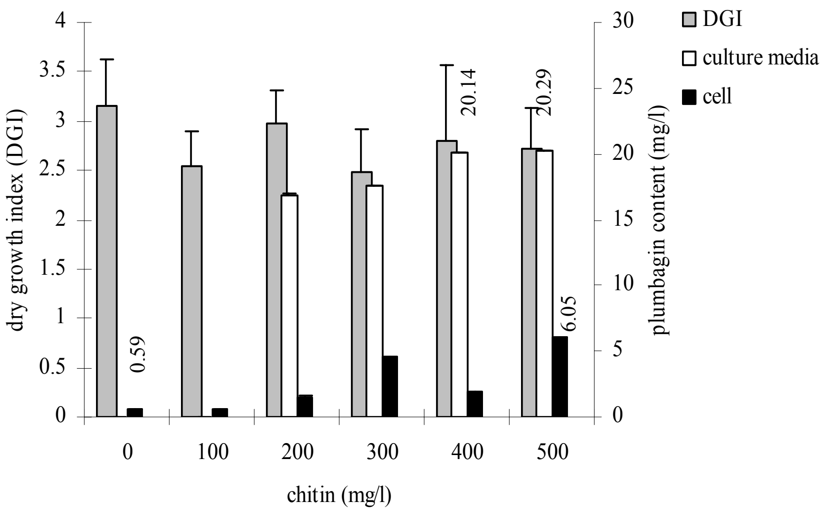

Figure 40 Effect of chitin at different concentrations on growth and the accumulation and release of plumbagin in cell suspension culture of *Plumbago indica* L. on 3rd day after elicitation.

5.3.2 Elicitation with Chitosan

Chitosan elicitor has been proven recently to significantly improve accumulation and stimulation biosynthesis of secondary metabolites in plant cell suspension cultures (Cheng *et al*., 2006).

The cells treated with chitosan changed the color from gray to yellow when they were elicited with 200-500 mg/l chitosan (which could be seen on 2^{th} day after elicitation) (Figure 41). The cell growth was decreased when increased concentrations of chitosan especially over 200 mg/l chitosan (Figure 42). The cell cultures treated with 0.1% acetic acid (control) had a few effect on releasing the plumbagin while the cell treated with water (control) did not release the plumbagin.

Plumbagin of 18.35 mg/l was released into liquid medium 2 days after adding 200 mg/l chitosan. This chitosan concentration was optimum for the induction of the secondary metabolites which has been reported on indirubin production from suspension culture of *Polygonium tinctorum* (Kim *et al*., 1997) and also has been found to be the most effective in increasing of plumbagin accumulation in suspension cultures of *Plumbago rosea* (Komaraiah *et al*., 2003). The plumbagin was not released into the medium in unelicited cell (control).

However, yields of plumbagin elicited with chitin and chitosan in cell suspension culture were much lower than those of hairy root cultures elicited with chitin (75.05 mg/l) and with chitosan (46.88 mg/l).

0.1% acetic acid **H_2O** **100** **200** **300** **400** **500**

chitosan (mg/l)

Figure 41 The change of cell color from gray to yellow color after chitosan elicitation at different concentrations in *Plumbago indica* L. cell suspension culture.

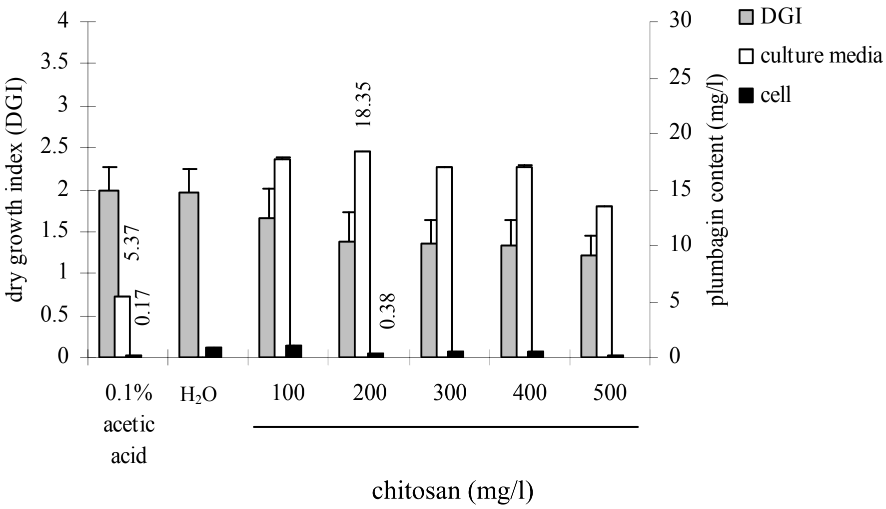

Figure 42 Effect of chitosan at different concentrations on growth, the accumulation and release of plumbagin in cell suspension cultures of *Plumbago indica* L. on 2nd day after elicitation.

5.3.3 Effect of Chitin and Chitosan on Cell Death of *P. indica* L. Cell Culture

It is believed that the stronger stimulation of secondary metabolites by biotic elicitor usually occurred in the late exponential growth stage of plant cell culture (Buitelar *et al*., 1992). In this experiment, 12-day-old *P. indica* L. cell suspension cultures were treated with different concentrations of chitin or chitosan (100-500 mg/l). There are two controls for chitosan treated cells; 0.1 % acetic acid. and the culture media. Cells were analyzed for viability after incubation using Evan's blue staining assay. Evans blue dye is excluded from viable cells and dead cells that retain intact plasma membranes. The dye absorbed by dead cell was latter extracted and subjected to quantitative analysis by optical absorbance procedure at OD_{600}. The more dead cells, the more dye absorbed and resulted in the higher OD_{600} value. As shown in Figure 43, OD_{600} of the control treatments (non-elicited cells) of chitin was 0.62 and chitosan was 0.67. The OD_{600} of acetic acid treatments and non-stain cells was 0.49 and 0.963, respectively. Cell viability was reduced after the addition of elicitors as indicated by higher OD_{600} The more elicitor added, the higher OD_{600} value obtained.

This result indicated that treatment of suspension culture cells with relatively high chitin or chitosan concentrations led to cell death. Cell death caused by chitosan could be considered as leakage from a relatively small compartment, i.e., cytosol. Long term permeabilization with chitosan showed a time dependent plumbagin released from *P. indica* L. cells into the culture medium at various chitin or chitosan concentrations. Increased chitin or chitosan concentrations caused more cell death. Some plant cell cultures treated with chitosan such as *Chenopodium rubrum*, the pigment of amaranthin was released during 5 h of treatment, which was correlated with cell death. Pore formation on the plasmalemma can also be related to the degree of deacetylation of chitosans. Highly charged chitosan polymers induced the higher degree of pore formation on plasmalemma (Dörnenburg and Knorr, 1995). This means that a critical charge density existed which led to loss of cell viability and initiated the diffusion of plumbagin from *P. indica* L. cells.

The internal control with acetic acid (OD_{600}=0.5) showed no significant in cell death compared to the control treatments. This is due to the medium was adjusted to 5.7. However, the lower pH level has been observed the release of secondary metabolites and growth inhibition.

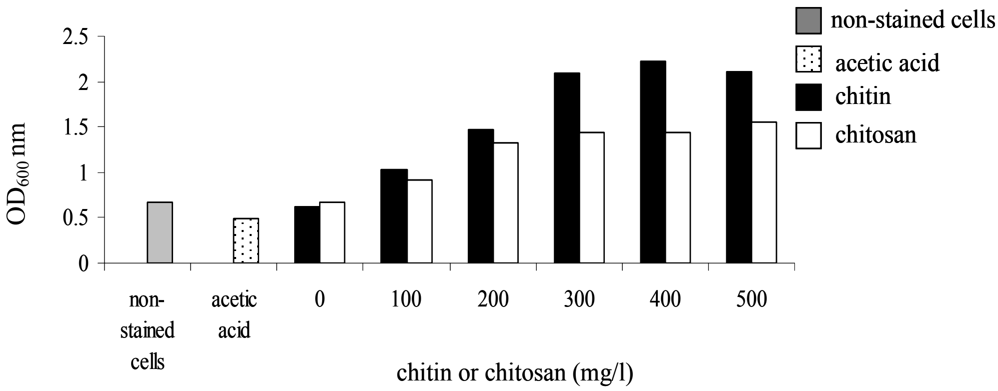

Figure 43 Effects of chitin and chitosan elicitors on cell death in *Plumbago indica* L. cell suspension culture.

5.4 Other Elicitors

5.4.1 Effects of Yeast Extract on Growth and Plumbagin Production in *P. indica* L. Cell Suspension Cultures

Growth of the *P. indica* L. cell suspension cultures was not affected by addition of yeast extract. However, the precipitated yeast extract had slight effect on cell growth. The precipitated yeast extract at 100 mg/l increased hairy root growth which DGI was up to 3.03 and then decreased with increased the concentrations of elicitor.

Production of plumbagin after yeast extract precipitated elicitation was observed. However, trace amount of plumbagin was found in both elicited cells and culture media. Cell suspension cultures treated with precipitated yeast extract were found to release plumbagin into the media in higher amount than the treatment of non-precipitated yeast extract. The highest plumbagin content of only 3.74 mg/l was obtained from the cultures elicited by 250 mg/l precipitated yeast extract (Figure 44).

Yeast elicitor is one of the most commonly used elicitors for enhancement the secondary metabolites. Yeast extract was known to effectively bind to receptors on the plant cells. It induced the synthesis of phenylalanine ammonium lyase activity and enhanced secondary metabolites accumulation in plant cell cultures of *Cistanche deserticola* (Cheng *et al*., 2005). However, not all plant species response to this elicitor. In some case, yeast elicitor reduced the secondary metabolite such as camptothecin production in cell suspension cultures of *Ophiorrhriza pumila*, (Saito *et al*., 2001). Yeast extract used as elicitor in *P. indica* L. cells did not improve the plumbagin production. Although, in some concentrations had effect on release the plumbagin into culture media.

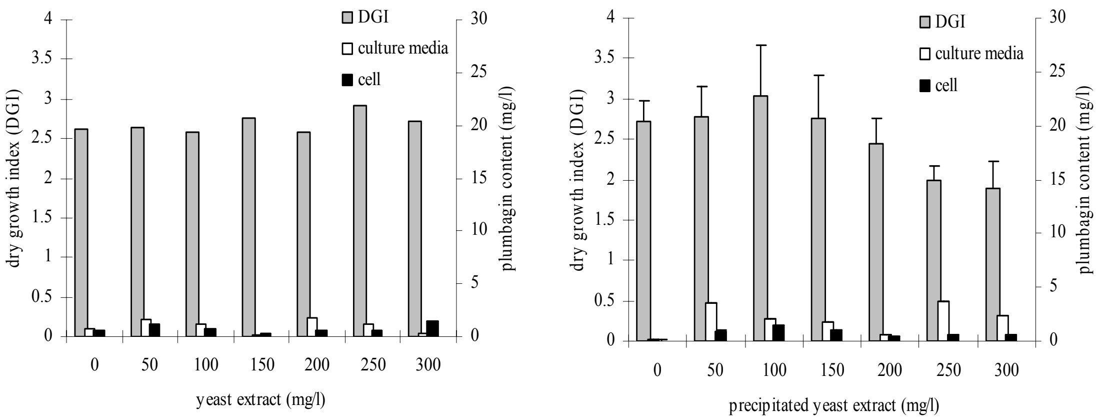

Figure 44 Cell growth and plumbagin production in *Plumbago indica* L. cell suspension cultures after elicited with yeast extract (A) and precipitated yeast extract (B).

5.4.2 Effects of Fungal Mycelium Elicitor on Growth and Plumbagin Production in *P. indica* L. Cell Suspension Cultures

Zhao *et al*. (2001) used various kinds of fungal elicitor on *Chatarantus roseus* and gained 2 to 5-fold increment of ajamalicine, serpentine and catharanthine. Similarly, Sirvent and Gibson (2002) also successfully used *Collectotrichum gloeosporioides* and obtained two folds of hypercins and hyperforin from *Hypericum perforatum* L. cell. In this research, homogenized mycelium of *Collectotrichum capsici* was used as elicitor in *P. indica* L. cell suspension culture. The growth of the *P. indica* L. cell suspension cultures had been slightly effected after addition of fungal elicitor. The fungal elicitor was not promoted the release of plumbagin but it slightly increased plumbagin accumulation in the cells when the concentrations of fungal mycelium increased (Figure 45). The cell color changed into pale yellow when high concentrations of fungal mycelium (400-500 mg/l) applied (Figure 46). The cell walls of higher fungi such as the genus *Colletotrichum* are composed of chitin. There has been reported on used a lectin probe to study the distribution of chitin in the walls of *C. acutatum and C. fragariae* during the initial infection of the strawberry host (Curry *et al*., 2002). *Hypericum perforatum* L. cell suspension culture elicited with fungal biomass of *C. gloeosporioides* at the concentration of 250 mg/l increased seven folds in xanthone accumulation (Conceição *et al*., 2006). The *C. capsici* mycelia at 500 mg/l increased 4.94 folds in plumbagin production. It could be suggested that *C. capsici* mycelia at higher concentration than 500 mg/l may increase the plumbagin production as pure chitin elicitor. However, the specific effects of fungal elicitors are due to the specificity of the interactions between the fungi elicitor signals and plant cell receptors, and the complexity of elicitor signal transduction and thereafter defense responses in plant cells (Zhao *et al*., 2001).

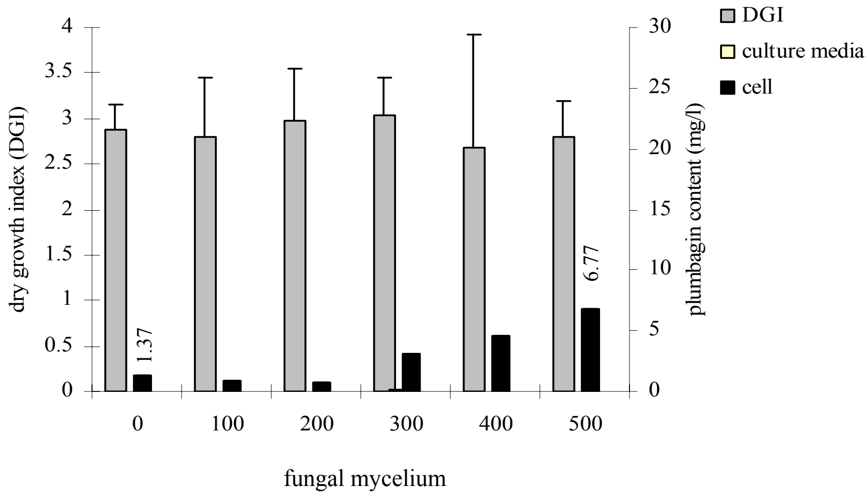

Figure 45 Effect of *Collectotrichum capsici* mycelium at different concentrations on *Plumbago indica* L. cell growth and on the accumulation and release of plumbagin in cell suspension cultures on day 7th after elicitation

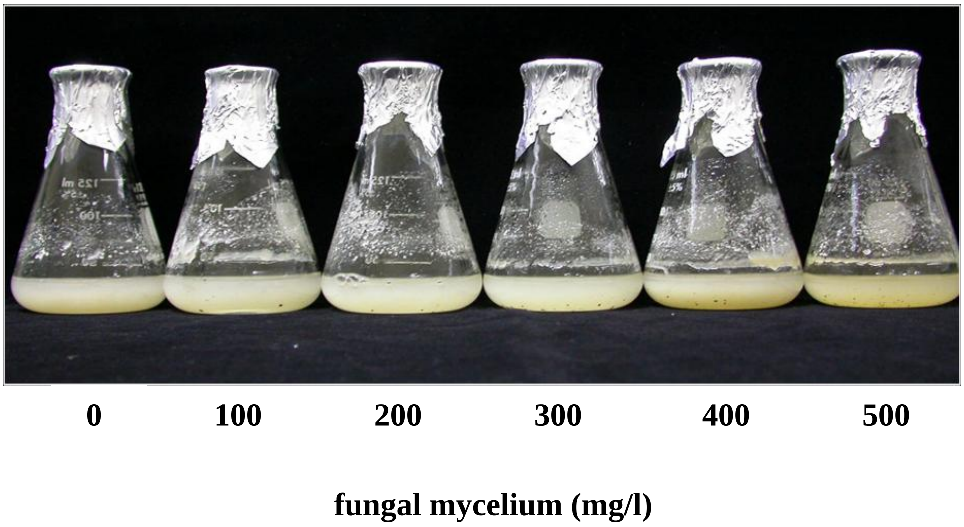

Figure 46 The *Plumbago indica* L. cell suspension culture cells turned to pale yellow after elicitation with *Collectotrichum capsici* mycelium at different concentrations.

5.5 Time of Exposure to Elicitors in Cell Suspension Culture

Time course of cell growth and plumbagin accumulation of *P. indica* L. cell cultures treated by 200 mg/l chitosan or 300 mg/l chitin were shown in Figure 47. Cell cultures treated with chitosan decreased in DGI compared to cell cultures treated with chitin. The cell cultures treated with chitin released plumbagin into liquid medium on day 3rd while in chitosan treatment, plumbagin was released on day 2nd after elicitation. The maximum total plumbagin content from cell cultures elicited with chitin was 24.29 mg/l, in which 16.17 mg/l in liquid medium and 8.12 mg/l in cell. The maximum plumbagin content in cell cultures elicited with chitosan was only 11.27 mg/l in liquid medium plus trace amount in cell.

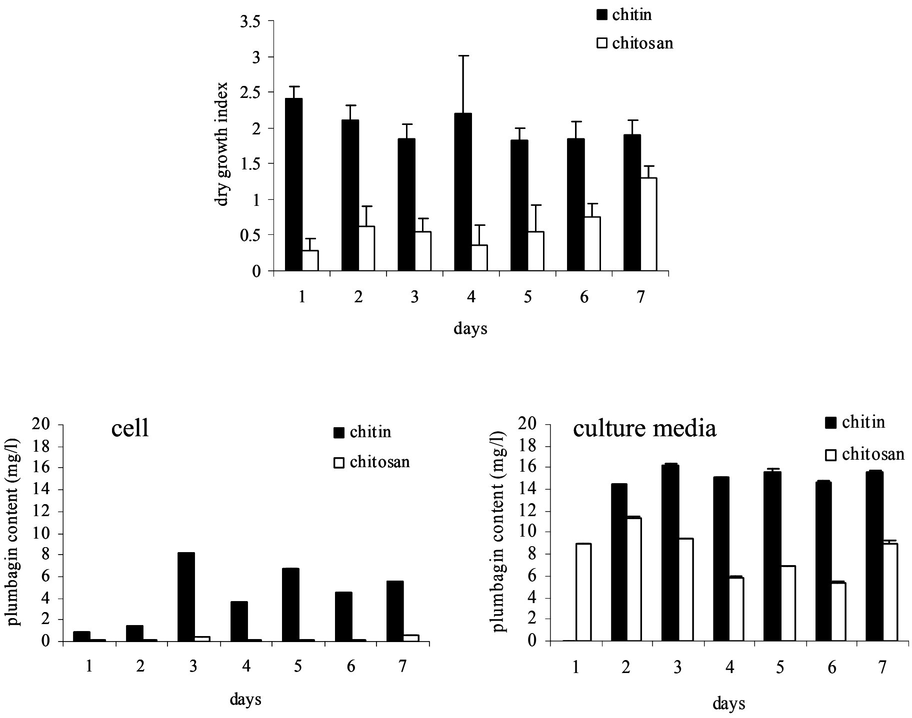

Figure 47 Dry growth index and plumbagin content (mg/l) in cell suspension culture of *Plumbago indica* L. harvested at different time of elicitation with 200 mg/l chitosan.

5.6 Growth and Plumbagin Accumulation of *P. indica* L. Hairy Root and Cell Suspension Cultures Multiple Treated by Chitosan

The time course experiment, the effect of chitosan elicitor on plumbagin release into the medium was seen on 5 days after elicitation, and the plumbagin was more released into the medium than chitin elicitation. Therefore, chitosan was selected for studying the duration of elicitation effect on secondary metabolite production. Four hundred mg/l chitosan was added to the 21-day-old hairy root culture, then at day 5, the medium was removed and the fresh media contained the same elicitor was added. This consequent elicitation was done on the same hairy root for five times started on days 21, 26, 31, 36 and 41 while the control treatment, the fresh medium without elicitor was used. The effects of repeated elicitation on plumbagin released into the media for both hairy root and cell suspension cultures are summarized in Figure 48. The maximum plumbagin content in hairy root cultures treated once by chitosan released into the media after 5 days of elicitation and then decreased rapidly to a low level in the subsequence elicitation in both 1/2MS and 1/2B5 media.This result indicated that single elicitation of chitosan is better than multiple elicitations.

In case of cell suspension culture, chitosan elicitor at the dose of 200 mg/l repeatedly added to 12-day-old cell suspension culture was improve plumbagin production in the second elicitation up to 31.19 mg/l and then rapidly decreased (Figure 48). The repeated addition of chitosan elicitor also led to inhibition of cell growth and later on, 100% cell death observed by Evan's blue assay (data not shown). However, in *Cistanche deserticola*, the repeated addition of chitosan led to slightly inhibit on cell growth but increased of phenylethanoid glycosides biosynthesis (Cheng *et al*., 2006). This difference may accounte for the genetic background of different plant materials.

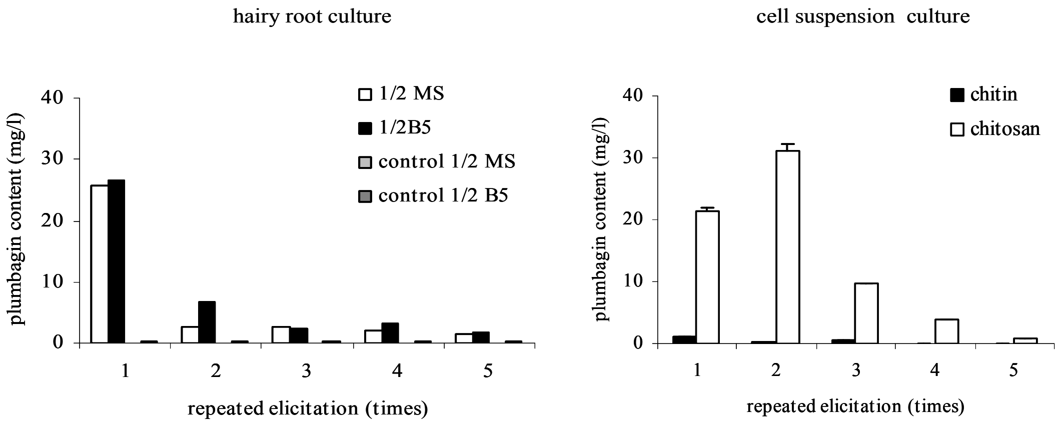

Figure 48 Plumbagin content in *Plumbago indica* L. hairy root and cell suspension cultures with multiple treated by chitosan.

5.7 Comparison of Plumbagin Production after Elicited with Various Elicitors in Hairy Root and Cell Suspension Cultures

In all elicitation experiments mention earlier, hairy root culture in combination with elicitors showed that plumbagin accumulation depend on type of culture media and the elicitors. Chitin and chitosan were suitable elicitors for stimulation the release of plumbagin into the culture media. The majority of hairy roots growth after treated with chitin yielded higher DGI than hairy roots treated with chitosan. However, addition of chitin or chitosan in hairy root culture resulted in increased plumbagin production which was slightly different between these two elicitors, approximately 3 folds for elicitation with chitin and 3-4 folds for elicitation with chitosan. The secretion of plumbagin also depends on acetic acid which used as solvent for chitosan solution but it slightly effect on plumbagin accumulation. In the other hand, yeast extract and fungal mycelium did not stimulate the release of plumbagin into culture media. The highest plumbagin content data obtained from the elicitation by various elicitors was shown in the Figure 49. The plumbagin accumulation was high in hairy roots treated with precipitated yeast extract or fungal mycelium of *C. capsici*. By comparison, among chitin, chitosan and fungal mycelium elicitors show no difference.

Cell suspension cultures in combination with elicitors showed that plumbagin production depended on the type of elicitor used. The elicitation of plumbagin by various elicitors was summarized in the Figure 49. The release of plumbagin in each of treat cultures of different elicitors shown in Figure 50. The data were the highest plumbagin content obtained from each elicitor. The addition of chitin or chitosan into culture media resulted in more increase of plumbagin production and more release of plumbagin into the culture medium than other elicitors. The plumbagin was slightly accumulated in cell culture after elicited with chitosan while chitin increased the plumbagin accumulation in cell. Acetic acid which used for chitosan preparation was also inserted its effect on the release of plumbagin into the media. Then, the secretion of plumbagin depends on both chitosan and acetic acid and depends upon cell viability which is reduced after the addition of chitin or chitosan as well. In comparision, chitin has to be used at much higher concentration than chitosan which consequently caused the higher cost of elicitation. Moreover, the preparation of chitin was quite inconvenience. Thus chitosan, in these senses, showed superior elicitation than that of chitin. Similar results have been reported its enhancement of secondary metabolites production in various medicinal plants such as *Cistanche deserticola* (Cheng *et al.*, 2006), *Taxus chinensis* (Luo and He, 2004) and *Rubia tinctorum* (Vasconsuelo, 2004). The other elicitors tested, fungal mycelium and yeast extract, were less effective for plumbagin elicitation.

These elicitation experiments indicated that not all plant cell cultures were responds to all elicitors. In some plant, chitosan has no effect on stimulating the synthesis of glucotropaeolin from *Farsetia aegyptia* cell suspension. If chitosan was used in combination with other elicitor such as methyl jusmonate it inhibited the stimulatory effect which possibility due to increassing cell permeability (Al-Gendy and Lockwood, 2005).

Decision to choose the elicitor for stimulating the plumbagin production in hairy root and cell suspension cultures depends on factors concerned as shown in Table 7. It can be suggested that chitosan is a good elicitor and advantage because its effect on cell permeabilization and not take long time for elicitation. Morever, chitosan is cheaper and easier to prepare than chitin. In the study, chitosan is effects on the release of plumbagin into the medium in both hairy root and cell suspension cultures. It is easy to extract the plumbagin from the culture medium.

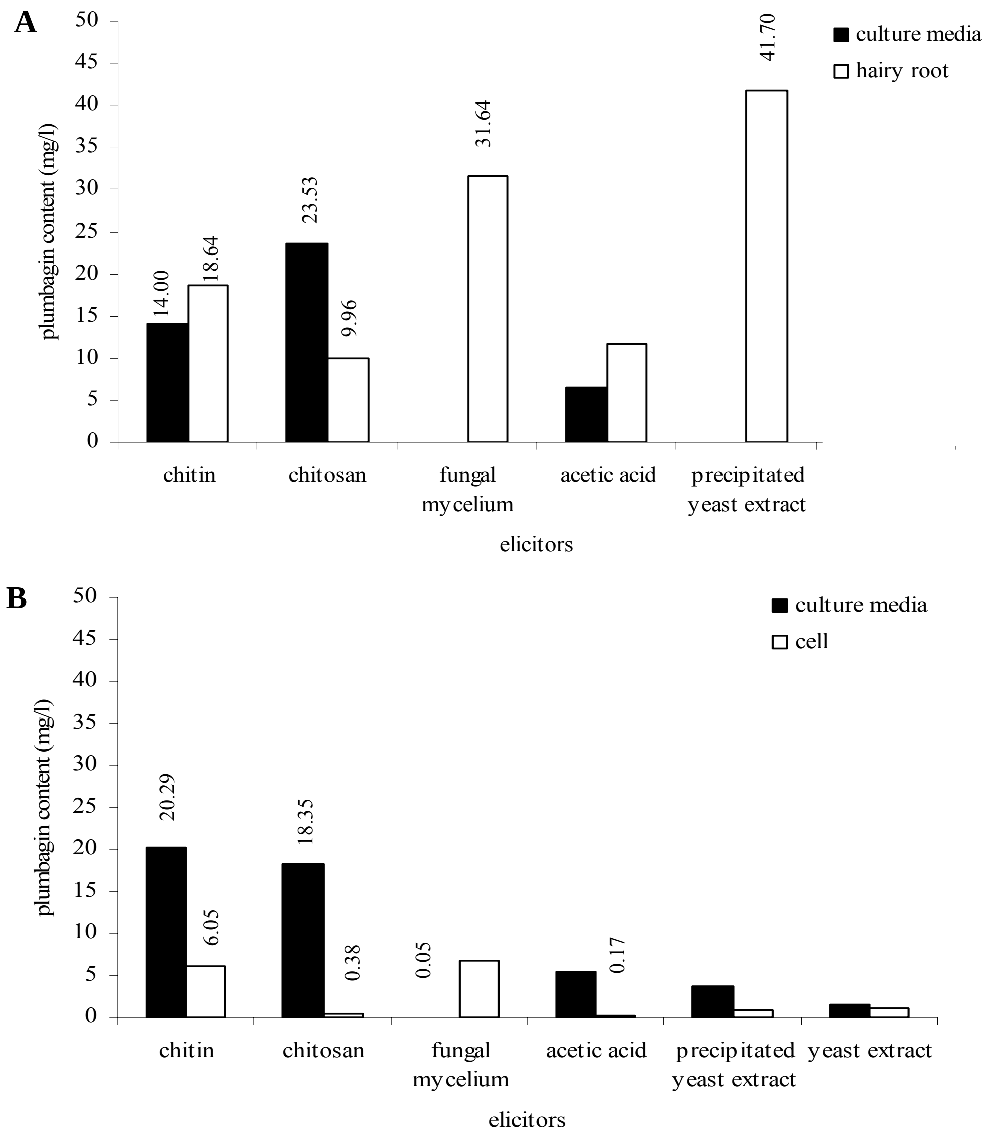

Figure 49 Plumbagin content in cells and culture media of *Plumbago indica* L. hairy root (A) and cell suspension cultures (B) after treated with various elicitors.

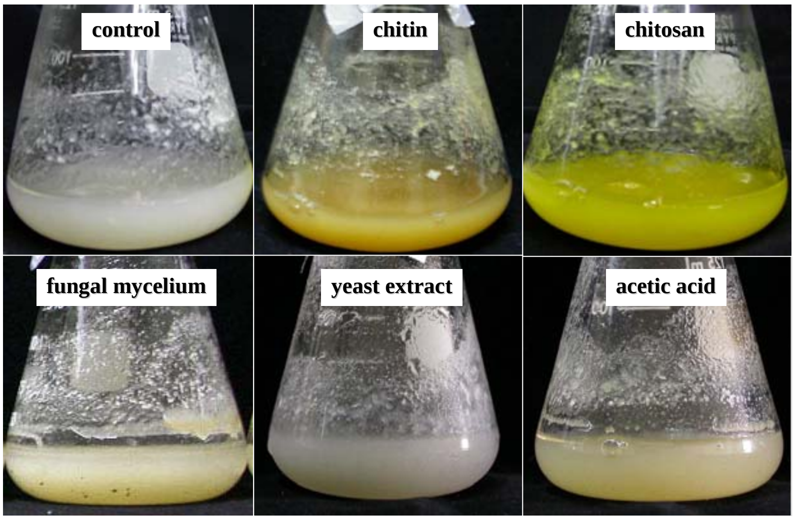

Figure 50 The change of cell color after elicitation with different elicitors in *Plumbago indica* L. cell suspension culture

Table 7 Comparison of various elicitors for plumbagin stimulation

Elicitors	Procedure	Dose (mg/l)	Duration stimulation (days)		Plumbagin Releasing*	Price (Baht)
			Hairy root cultures	Cell suspension cultures		
1. chitin	complicate	400-500	>8	3	stimulation	3,000 / 1 g
2. chitosan	simple	200-300	5	2	stimulation	1,700 / 25g
3. fungal mycelium (*Colletotrichum capsici*)	complicate	>500	-	-	no stimulation	-
4. acetic acid	simple	0.06%	5	2	slightly affect	350 / 2.5 l
5. precipitated yeast extract	complicate	50	>7	>7	slightly affect	2,500 / 500 g
6. yeast extract	simple	200	>7	>7	slightly affect	2,500 / 500 g

* The release of plumbagin into culture medium

CONCLUSIONS

In this reseach, the immunological technique was used for localization of plumbagin. Plumbagin-BSA conjugate was successfully prepared. The antibody raised against this conjugate elicited immune response in rabbit better than in BALC/c mice with the titers of 625 compared to 25, respectively. Based on this result, the rabbit antiserum was selected for the immunohistochemical study. Eventhough the antiserum had low titer, the immunolocalization study using fluorescein-labelled goat anti-rabbit IgG demonstrated the specific reactions with the plumbagin in *P. indica* L. plant tissues. The reactions indicated that the plumbagin was located in all plant parts tested such as leaf, stem, upper and lower parts of root where it was mainly found in cell membrane and intercellular space.

The preparation technique for plumbagin-carrier protein conjugates could be done under N_2 gas and low temperature. It was expected that N_2 gas might create the cross linking between a methyl group of plumbagin and an amine group in BSA.

Tissue preparation technique is important for localization of secondary metabolites. Plumbagin is easy to be degraded by heating because it is a volatile substance. It can be suggested that using low temperature in the step of tissue fixation prevents the lost of plumbagin.

The study also focused on the production of plumbagin from hairy root callus and cell suspension cultures of *P. indica* L.. The results showed that hairy roots were fast growing and yielded high plumbagin content in half-MS liquid media with 20 g/l sucrose. The suitable liquid medium for proliferate callus and cell suspension of *P. indica* L. was MS + B5 vitamins supplemented with 0.2 mg/l NAA, 0.2 mg/l 2,4-D and 0.4-0.5 mg/l kinetin. The kinetin containing media yielded higher dry weight of callus and cell suspension and higher plumbagin content than BA containing media. The callus explant yielded the lowest plumbagin content among the tested cultures. Cell suspension culture under the dark condition yielded higher level of plumbagin than cell culture under the light condition. The root-derived cell suspension culture yielded higher plumbagin than the internode-derived. Interestingly, the growth pattern of cell suspension culture could be devided into two types: aggregated cells and suspension cells. These characters could be found in all explant types. Moreover, the analysis of plumbagin content revealed only trace amount of plumbagin in the aggregated cells while the much higher amount was found in the suspension cells.

Hairy root and cell suspension cultures were treated with biotic elicitors (chitin, chitosan, fungal mycelium and yeast elicitor) and abiotic elicitors (acetic acid) were conducted to improve the production of plumbagin. Chitin and chitosan induced the accumulation and release of the plumbagin in the hairy root and cell suspension cultures. The release of the plumbagin in cell suspension cultures was stimulated on the second day after cells were treated with chitosan and on the third day after chitin treatment. On the other hand, the release of plumbagin in hairy root cultures was first observed on the fifth and eighth day after chitosan and chitin treatment, respectively. The addition of acetic acid slightly affected the release of the plumbagin but less significant than chitosan. The treatment with chitin (400-500 mg/l) or chitosan (300 mg/l) induced 3-4 folds increase in the plumbagin content in hairy roots. Chitin (400-500 mg/l) induced up to 20 folds while chitosan (200 mg/l) induced 4 folds increase in the plumbagin content in cell suspension culture. Fungal mycelium elicitor reduced plumbagin accumulation in hairy roots but increased its content in cell suspension culture. The precipitated yeast extract can not stimulate the release of plumbagin into the culture media of hairy root culture and only little secretion of plumbagin in cell suspension cultures was observed. It is note that 50-150 mg/l precipitated yeast extract yielded higher plumbagin accumulation in hairy roots than in cell cultures.

LITERATURE CITED

Adamczyk, M., J.C. Gebler and P.G. Mattingly. 1996. Characterization of protein – hapten conjugates: 2. Electrospray mass spectrometry of bovine serum albumin-hapten conjugates. **Bioconjugate Chem**. 7: 475.

Ahmed, K.M., F.E. Kandil, and T.J. Mabry. 1999. An anticancer tannin and other phenolics from *Limonium axillare* (Fam. Plumbaginaceae). **Asian J. of Chem.** 11: 261-263.

Al-Gendy, A.A. and G.B. Lockwood. 2005. Production of glucosinolate hydrolysis products in *Farsetia aegyptia* suspension cultures following elicitation. **Fitoterapia** 76: 288-295.

Anke, S., D. Niemüller, S. Moll, R. Hänsch and D. Ober. 2004. Polyphyletic origin of pyrrolizidine alkaloids within the Asteraceae, evidence from differential tissue expression of homospermidine synthasse. **Plant Physiol.** 136: 4037-4047.

Beale, M.H. 1999. Immunological methods in plant hormone research, pp. 61-88. *In* P.J.J. Hooykaas, M.A. Hall and K.R. Libbenga, eds., **Biochemistry and Molecular Biology of Plant Hormone.** Elsevier Science.

Benhamou, N. 1996. Elicitor-induced plant defence pathways, **Trends in Plant Sci.** 1: 233-240.

Bergman, R.A., A.K. Afifi and P. M. Heidger. 1996. **Histology**. W.B. Saunders Company.

Bhargava, S.K. 1984. Effects of plumbagin on reproductive function of male dog. **Ind. J. Exp Biol.** 22: 153-156.

Bhagwath, S.G. 1997. **Optimization and Scale-Up of Thiarubrine A Production from Hairy Root Cultures of *Ambrosia artemisiifolia*.** Dissertation, Louisiana State University.

Bhagwath, S.G. and M.A. Hjortsø. 2000. Statistical analysis of elicitation strategies for thiarubrine a production in hairy root cultures of *Ambrosia artemisiifolia*. **J. Biotech.** 80: 159-167.

Bolta, ž, D. Baričevič, B. Bohanec and S. Andrenšek. 2000. A preliminary investigation of ursolic acid in cells suspension culture of *Salvia officinalis*. **Plant Cell, Tiss. Org. Cult.** 62: 57-63.

Botanical Dermatology Database. 1999a. *Plumbaginaceae* (date of last modification, 1979). Available Source: http://bodd.cf.ac.uk/BotDermfolder/ BotDermP/ PLUM.html, December 25, 2001.

Botanical Dermatology Database. 1999b. *Droseraceae (Sundew* family)(date of last modification, 1984). Available Source: http://bodd.cf.ac.uk/BotDermfolder /BotDermP/DROS.html, December 25, 2001.

Bourgaud, F., A. Gravot, S. Milesi and E. Gontier. 2001. Production of plant secondary metabolites: a historical perspective. **Plant Sci.** 161: 839- 851.

Brisson, L., P.M. Charest, V. De Luca and R.K. Ibrahim. 1992. Immunocytochemistry localization of vindoline in mesophyl protoplasts of *Chatharanthus roseus*. **Phytochem.** 31: 465- 470.

Brigham, L.A., P.J. Michaels and H.E. Flores. 1999. Cell-specific productiion and antimicrobial activity of naphthoquinones in roots of *Lithospermum erythrorhizon*. **Plant Physiol.** 119: 417- 428.

Brodelius, P., C. Funk, A. Haner and M. Villegas. 1989. A procedure for the determination of optimal chitosan concentrations for elicitation of cultured plant cells. **Phytochem.** 28: 2651-2654.

Budzianowski, J. 2000. Naphthoquinone glucosides of *Drosera gigantia* from *in vitro* cultures. **Planta Med.** 66: 667- 669.

Buitelar R.M. and J. Tramper. 1992. Strategies to improve the production of secondary metabolites with plant cell cultures: a literature review. **J. Biotech.** 23: 111-141.

Canto-Canché, B.B., A.H. Meijer, G. Collu, R. Verpoorte and V.M. Loyola-Vargas. 2005. Characterization of a polyclonal antiserum against the monoterpene monooxygenase, geraniol 10-hydroxylase from *Catharanthus roseus*. **J. Plant Physiol.** 162: 393- 402.

Chan, E.C.Y. and P.C. Ho. 2002. Preparation and characterization of immunogens for antibody production against metanephrine and normetanephrine. **J. Immuno. Methods.** 226: 143-154.

Chanprame, S., S. Tatreerod, P. Saralamp and S. Chanprame. 2003. High efficiency system for micropropagation of *Plumbago indica* L. pp. 263 *In* **The 3 rd World Congress on Medicinal and Aromatic plants for Human Welfare**. Ching Mai, Thailand.

Chawla, H.S. 2000. **Introduction to Plant Biotechnology**. Science Publisher, Inc. Plymouth, UK.

Chen, H. and F. Chen. 2000. Effects of yeast elicitor on the growth and secondary metabolism of high-tanshinone-producing line of the Ti transformed *Salvia miltiorrhiza* cells in suspension culture. **Process. Biochem.** 35: 837-840.

Chen, H., F. Chen, F.C.K. Chiu and C.M.Y. Lo. 2001. The effect of yeast elicitor on the growth and secondary metabolism of hairy root cultures of *Salvia miltiorrhiza*. **Enzyme Microb. Technol.** 28: 100-105.

Cheng, H.Y., B. Guo, H.Y. Zhou, W. Ni and C. Z. Liu. 2005. Repeated elicitation enhances phenylethanoid glycosides accumulation in cell suspension cultures of *Cistanche deserticola*. **Biochem. Eng. J.** 24: 203-207.

Cheng, X.Y., H.Y. Zhou, X. Cui, W. Ni and C.Z. Liu. 2006. Improvement of phenylethanoid glycosides biosynthesis in *Cistanche deserticola* cell suspension cultures by chitosan elicitor. **J. Biotech.** 121: 253-260.

Chilton, M.D., D.A. Tepfer, A. Petit, C. David and F. Casse-Delbart. 1982. *Agrobacterium rhizogenes* inserts T-DNA into the genomes of the host plant root cells. **Nature** 295: 432-434.

Chong, T.M., A.M. Abdullah, O.M. Lai, F.M.N. Aini and N.H. Lajis. 2005. Effective elicitation factors in *Morinda elliptica* cell suspension culture. **Process. Biochem.** 40: 3397-3405.

Choosakul, O. 2000. **Plumbagin Production in Cell Suspension Cultures of *Plumbago zeylanica* L.** M.S. Thesis. Chulalongkorn University, Thailand.

Clausen, C.A. and F. Green III. 1997. Antibody-mediated immunochemistry and immunoassay in plant-related diseases. pp 73-78. *In* Dashek, William V., ed. **Method in Plant Biochemistry and Molecular Biology.** Boca Raton, CRC Press.

Collin, H.A. and S. Edwards. 1998. **Plant Cell Culture**. BIOS Scientific Publishers Limited.

Conceição, L.F.R., F. Ferreres, R.M. Tavares and A.C.P. Dias. 2006. Induction of phenolic compounds in *Hypericum perforatum* L. cells by *Colletotrichum gloeosporioides* elicitation. **Phytochem.** 67: 149-155.

Crouch, I.J., J.F. Finnie and J. Staden. 1990. Studies on the isolation of plumbagin from *in vitro* and *in vivo* grown *Drosera* species. **Plant Cell, Tiss. Org. Cult.** 21: 79-82.

Curry, K.J., M. Abril, J.B. Avant and B.J. Smith. 2002. Strawberry anthracnose: histopathology of *Colletotrichum acutatum* and *C. fragariae*. **Phytopathol.** 92: 1055-1063.

Dankwardt, A. and B. Hock. 2001. Immunolocalization of non-extractable (bound) residues of pesticides and industrial contaminants in plants and soil. **Chemosphere** 45: 523-533.

Darvill, A.G. and P. Albersheim. 1984. Phytoalexins and their elicitors-defense against microbial infection in plants. **Ann. Rev. Plant Physiol.** 35: 243-275.

Das, G. and G.R. Rout. 2002. Direct plant regeneration from leaf explants of *Plumbago* species. **Plant Cell, Tiss. Org. Cult.** 68: 311-314.

Desta, B. 1993. Ethiopian traditional herbal drugs. Part II. Antimicrobial activity of 63 medicinal plants. **J. Ethnophramacol.** 39: 129-139.

Didry, N., L. Dubrevil and M. Pinkas. 1994. Activity of anthoquinonic and naphthoquinonic compounds on oral bacteria. **Pharmazie.** 49: 681-683.

Dong, H.D. and J.J. Zhong. 2002. Enhanced taxane productivity in bioreactor cultivation of *Taxus chinensis* cells by combinding elicitatin, sucrose feeding and ethylene incorporation. **Enzyme Microb. Technol.** 31: 116-121.

Dörnenburg, H. and D. Knorr. 1995. Strategies for the improvement of secondary metabolite production in plant cell cultures. **Enzyme Microb. Technol.** 17: 674-684.

Durand, R. and M.H. Zenk. 1971. Biosynthesis of plumbagin (5-hydroxy-2-methyl-1,-4-naphthoquinone) via the acetate pathway in higher plants. **Tetrahedron Lett.** 32: 3009-3012.

Edwards, R. 1996. **Immunoassays. : Essential Data.** BIOS Scientific Publishers Limited.

Ertola, R.J. and R. Hours. 1998. Role of yeast extract components in microbial cultures not associated with aminoacids, vitamins and minerals: a review. **Appl. Biol. Sci.** 4: 1-15. Cited S.I Pitta-Alvarez, T.C. Spollansky and A.M. Giulietti. 2000. The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of *Brugmansia candida*. **Enzyme Microb. Technol.** 26: 252-258.

Farnsworth 1999. Napralert profile for *Plumbago rosea*. (Computer Program). Illinois: The Board of trustees of the University of Illinois.

Figueiredo, S.F.L., C. Simões, N. Albarello and V.R.C. Viana. 2000. *Rollinia mucosa* cell suspension culture: Establishment and growth conditions. **Plant Cell, Tiss. Org. Cult.** 63: 85-92.

Fisher, D.B. 1972. The retention of water-soluble compounds during freeze substitution and microautoradiography. **Plant Physiol.** 49: 166-171.

Fu, X.Q. and D.W. Lu. 1999. Stimulation of shikonin production by combined fungal elicitation and *in situ* extraction in suspension cultures of *Arnebia euchroma*. **Enzyme Microb. Technol.** 24: 243-246.

Fuller, KW. 1984. Chemicalsfrom plant cell cultures: some biochemical and physiological pointers. **Chem Ind**. 3: 825-833. Cited Zhao, D., Y. Huang, Z. Jin, W. Qu and D. Lu. 2003. Effect of aggregate size in cell cultures of *Saussurea medusa* on cell growth and jaceosidin production. **Plant Cell Rep.** 21: 1129-1133.

Gamborg, O.L., R.A. Miller and K. Ojimar. 1968. Nutrient requirements of suspension cultures of soybean root cell. **Exp. Cell Res.** 50: 151-158.

Ge, X and J. Wu. 2005. Tanshinone production and isoprenoid pathways in *Salvia miltiorrhiza* hairy rootsinduced by Ag^+ and yeast elicitor. **Plant Sci.** 168: 487-491.

Geissman, T.A. and D.H.G. Crout.. 1967. **Organic Chemistry of Secondary Metabolism.** San Francisgo, USA: Freeman, Cooper & Company.

Gelvin S.B. 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and intrigation. **Annu. Rev. Plant Physiol. Plant Mol. Biol.** 51: 223-256.

Giri, A., M. L. Narasu. 2000. Transgenic hairy roots: recent trends and applications. **Biotech. Adv.** 18: 1-22.

G´omez, L. G. 2004. Plant perception systems for pathogen recognition and defence. **Mol. Immuno.** 41: 1055–1062.

Grieve, M. 1995. Botanical. com. A modern Herbal. *Plumbago*. Available Source: http://www.botanical.com/botanical/mgmh/p/plumba54.html. December 25, 2001.

Gupta, M.M., R.K. Verma, G.C. Uniyal and S.P. Jain. 1993. Determination of plumbagin by normal phase high performance liquid chromatography. **J. Chromatogr.** 637: 209-212.

Harlow, E and D. Lane. 1988. **Antibodies: a Laboratory Manual.** Cold Spring Harbor Laboratory Press. New York.

Hatton, C.K. 1995. Pharmacognosy, Phytochemistry, Medicinal Plants. Intercept Ltd. UK.

Hermanson, G.T. 1996. **Bioconjugate Techniques.** Academic Press, New york.

Hook, I.L.I. 2001. Naphthoquinone contents of *in vitro* cultured plants and cell suspensions of *Dionaea muscipula* and *Drosera* species. **Plant Cell, Tiss. Org. Cult.** 67: 281-285.

Jacob, A. and N. Malpathak. 2004. Green hairy root cultures of *Solanum khasianum* Clarke-a new route to *in vitro* solasodine production. **Current Sci.** 87: 1142-1147.

Jin, J.H., J.H. Shin, J.H. Kim, I.S. Chung and H.J. Lee. 1999. Effects of chitosan elicitation and media components on the production of anthraquinone colorants in Madder (*Rubia akane* Nakai) cell culture. **Biotech. Bioprocess. Eng.** 4: 300-304.

Jirayama, K., S. Akashi, M. Furiya and D. kukuhara. 1990. Rapid confermation and revision of the primary structure of bovine serum albumin by ESMIS and FRIT LC/MS. **Biochem. Biophys. Res. Commun.** 173: 639. Cited Chan, E.C.Y. and P.C. Ho. 2002. Preparation and characterization of immunogens for antibody production against metanephrine and normetanephrine. **J. Immuno. Methods.** 226: 143-154.

Jung, D.W., J.M. Lee and C. K. Sung. 2002. Enzyme-linked immunosorbent assay for the determination of 20(*S*)-protopanaxatriol. **Anal. Chim. Acta.** 462: 157–163.

Kim, J.H., J.H. Shin, H.J. Lee, I.S. Chung, and H.J. Lee. 1997. Effect of chitosan on indirubin production from suspension culture of *Polygonium tinctorium.* **J. Ferment. Bioeng.** 83: 206-208.

Kim, Y., B.E. Wyslouzil and P.J. Weathers. 2002. Secondary metabolism of hairy root cultures in bioreactors. **In vitro Cell. Dev. Biol. Plant.** 38: 1-10.

Kitanov, G.M. and P.P. Pashankov. 1994. Quantitative investigation on dynamics of plumbagin in *Plumbago indica* L. root and herb by HPLC. **Pharmazie.** 49: 642.

Kittipongpatana, N., R.S. Hock and J.R. Porter. 1998. Production of solasodine by hairy root, callus and cell suspension cultures of *Solanum aviculare* Forst. **Plant Cell, Tiss. Org. Cult.** 52: 133-143.

Kneer, R., A.A. Poulev, A. Olesinski and I. Raskin. 1999. Characterization of the elicitor-induced biosysthesis and secretion of genistein from roots of *Lupinus luteus* L. **J. Expt. Bot.** 50: 1553-1559.

Knox, R.B. 1982. Immunology and the study of plant, pp.293-346. *In* J.J. Marchalonis and G.W. Warr, eds. **Antibody as a Tool : The Applications of Immunochemistry.** A Wiley-Interscience Plublication, New York.

Komaraiah, P., P.B. Kavi Kishor and S.V. Ramakrishna. 2001. Production of plumbagin from cell suspension cultures of *Plumbago rosea* L. **Biotech. Lett.** 23: 1269-1272.

Komaraiah, P., R. Naga Amrutha, P.B. Kavi Kishor and S.V. Ramakrishna. 2002. Elicitor enhanced production of plumbagin in suspension cultures of *Plumbago rosea* L. **Enzyme Microb. Technol.** 31: 634-639.

Komaraiah, P., S.V. Ramakrishna, P. Reddanna and P.B. Kavi Kishor. 2003. Enhance production of plumbagin in immobilized cells of *Plumbago rosea* by elicitation and *in situ* adsorption. **J. of Biotech.** 101: 181-187.

Krell, H. W. 1993. Plant tissue and cells. pp. 79-88. *In* R.F. Masseyeff, W.H. Albert and N.A. Staines. **Methods of Immunological Analysis. Vol III.** VCH Pub. Inc., New York.

Krewzaler, F and K. hahlbrock. 1976. Flovanoide glycosides from illuminated cell suspension cultures of *Petroselinum hortense*. **Phytochem.** 12: 1149-1152.

Krishnan, S. 2003. *Plumbago indica*. Available Source: http://Butterfly Pavilion Horticulture.htm, January 10, 2003.

Kubo, I., M. Uchida and J.A. Klocke. 1983. An insect ecdysis inhibitor from the African medicinal plant, *Plumbago capsensis* (Plumbaginaceae). **Agri. Biol. Chem.** 47: 911-913.

Lehninger, A.L., D.L. Nelson and M.M. Cox. 1993. **Principles of Biochemistry**, 2nd edition, New York. Worth Publisher.

Liso, R., M.C. De Tullio, S. Ciraci, R. Balestrini, N.L. Rocca, L. Bruno, A. Chiappetta, M.B. Bitonti, P. Bonfante and O. Arrigoni. 2004. Localization of ascorbic acid, ascorbic acid oxidase, and glutathione in roots of *Cucurbita maxima* L. **J. Expt. Bot.** 55: 2589-2597.

Luckner, M., B. Diettrich and W. Lerbs. 1980. Cellular compartmentation and channelling of secondary metabolism in microorganisms and higher plants. pp. 103-142. *In* L. Rein-hold, J.B. Harborne and T. Swain, eds. **Progress in Phytochemistry.** Vol 6. Pergamon, Oxford., UK.

Luczkiewicz, M. and D. GłÓd. 2003. Callus cultures of *Genista* plants-*in vitro* material producing high amounts of isoflavones of phytoestrogenic activity. **Plant Sci.** 165: 1101-1108.

Luczkiewicz, M., R. Zárate, W. D. Migas, P. Migas and R. Verpoorte. 2002. Production of pulchelin E in hairy root, callus and cell suspension cultures of *Rudbeckia hirta* L**. Plant Sci.** 163: 91-100.

Luo, J. and G.Y. He. 2004. Optimization of elicitors and precursors for paclitaxel production in cell suspension culture of *Taxus chinensis* in the presence of nutrient feeding. **Process. Biochem.** 39: 1073-1079.

Mahoney, N., R.J. Molyneux and B.C. Campbell. 2000. Regulation of aflatoxin production by naphthoquinones of walnut (*Juglans regia*). **J. Agri. Food Chem.** 48: 4418-4421.

McCue, K.F.. 1990. Induction of Shikimic acid pathway enzymes by light in suspension cultured cells of Parsley (*Petroselinum crispum*). **Plant Physiol.** 94: 507-510.

McDonald, K.A. and A.P. Jackman. 1989. Bioreactor studies of growth and nutrient utilization in Alfalfa suspenion cultures. **Plant Cell Rep.** 8: 455-458.

Merkli, A., P. Christen and I. Kapetanidis. 1997. Production of diosgenin by hairy root cultures of *Trigonella foenum-graecum* L. **Plant Cell Rep.** 16: 632-636.

Mukundan, U. and M.A. Hjottso. 1991. Effect of light on growth and thiopene accumulation in transformed roots of *Tagetes patula.* **Plant Physiol.** 138: 252- 255.

Murashige, T. 1977. Manipulation of organ culture in plant tissue culture. **Botanical Bull. Acad. Sinica.** 18: 1-24. Cited G.R. Rout, S. Samantaray and P. Das. 2000. *In vitro* manipulation and propagation of medicinal plants. **Biotechnology Adv.** 18: 91-120.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. **Physiol. Plant.** 15: 473-479.

Nahálka, J., P. Blanárik, P. Gemeiner, E. Matúšová and I. Partlová. 1996a. The chemical/osmotic conditions for growth and plumbagin accumulation of *Drosophyllum lusitanicum* Link. suspension cultures. **Biotech. Lett.** 18: 1453-1458.

Nahálka, J., P. Blanárik, P. Gemeiner, E. Matúšová and I. Partlová. 1996b. Production of plumbagin by cell suspension cultures of *Drosophyllum lusitanicum* Link. **J. Biotech.** 49: 153-161.

Nahálka, J., J. Nahálková, P. Gemeiner and P. Blanárik. 1998. Elicitation of plumbagin by chitin and its release into the medium in *Drosophyllum lusitanicum* Link. suspension cultures. **Biotech. Lett.** 20: 841-845.

Nahálkova, J., F.O. Asiegbu, G. Daniel, J. Hŕib, B. Vookova, B. Pribulova and P. Gemeiner. 2001. Isolation and immunolocalization of a *Pinus nigra* lectin (PNL) during interaction with the necrotrops –*Heterobasidion annosum* and *Fusarium avenaceum.* **Physiol. Mol. Plant Pathol.** 59: 153-163.

Nakashima, J., T. Awano, K. Takabe, M. Fujita and H. Saiki. 1997. Immunocytochemical localization of phenylalanine ammonia-lyase and cynamyl alcohol dehydrogenese in differentiating tracheary elements derived from *Zinnia* mesophyll cells. **Plant Cell Physiol.** 38: 113-123.

Okršlar, V., B. Štrukelj, S. Kreft, B. Bohanec and J. Žel. 2002. Micropropagation and hairy root culture of *Solanum laciniatum* Ait. **In vitro Cell. Develop. Biol.** 38: 352-357.

Ortmann, I., G. Sumowski, H. Bauknecht and B.M. Moerschbacher. 2004. Establishment of a reliable protocol for the quantification of an oxidative burst in suspension – cultured wheat cells upon elicitation. **Physiol. Mol. Plant Pathol.** 64: 227-232.

Paiva, S.R. de, M.R. Figueiredo, T.V. Aragão and M.A.C. Kaplan. 2003. Antimicrobial activity *in vitro* of plumbagin isolated from *Plumbago* species. **Mem. Inst. Oswaldo Curz.** 98: 959-961.

Palazón, J., R.M. Cusidó, M. Bonfill, A. Mallol, E. Moyano, C. Morales and M.T. Piňol, 2003. Elicitation of different *Panax ginseng* transformed root phenotypes for an improved ginsenoside production. **Plant Physiol. Biochem.** 41: 1019-1025.

Panichayupakaranant, P. and S. Tewtrakul. 2002. Plumbagin production by root cultures of *Plumbago rosea*. **E. J. Biotech.** 5: 228-232.

Parimala, R. and P. Sachdanandam. 1993. Effect of plumbagin on some glucose metabolizing enzymes studied in rats in experimental hepatoma. **Mol. Cell. Biochem.** 125: 59-63.

Pasqua, G., B. Monacelli, N.Mulinaci, S. Rinaldi, C. Giacchererini, M. Innocenti and F.F. Vinceri. 2005. The effect of growth regulators and sucrose on anthocyanin production in *Camptotheca acuminate* cell cultures. **Plant Physiol. Biochem.** 43: 293-298.

Pitta-Alvarez, S.I. and A.M. Guilietti. 1999. Influence of chitosan, acetic acid and citric acid on growth and tropane alkaloid production in transformed roots of *Brugmansia candida*: Effect of medium pH and growth phase. **Plant Cell, Tiss. Org. Cult.** 59: 31-38.

Pitta-Alvarez, S.I., T.C. Spollansky and A.M. Giulietti. 2000. The influence of different biotic and abiotic elicitors on the production and profile of tropane alkaloids in hairy root cultures of *Brugmansia candida*. **Enzyme Microb. Technol.** 26: 252-258.

Prasain, J.K., C.C. Wang and S. Barnes. 2004. Mass spectrometric methods for the determination of flavonoides in biological samples. **FreeRadic. Biol. Med.** 37: 1324-1350.

Premakumari, P., K. Rathinam and G. Santhakumari. 1977. Antifertility activity of plumbagin. **Ind. J. Med. Res.** 65: 829-838.

Qin, W.M. and W.Z. Lan. 2004. Fungal elicitor-induced cell death in *Taxus chinensis* suspension cells is mediated by ethylene and polyamines. **Plant Sci.** 166: 989-995.

Radman, R., T. Saez, C. Bucke and T. Keshavarz. 2003. Elicitation of plants and microbial systems. **Biotech. Appl. Biochem.** 37: 91-102.

Rajendran, L. G.A. Ravishankar, L.V. Venkataraman and K.R. Prathiba. 1992. Anthocyanin production in callus cultures of *Daucus carota* L. as influenced by nutrient stress and osmoticum. **Biotech. Lett.** 14: 707-714. Cited S. Ramachandra Rao and G.A. Ravishankar. 2002. Plant cell cultures: Chemical factories of secondary metabolites. **Biotech. Adv.** 20: 101-153.

Rao, S. and G.A. Ravishankar. 2002. Plant cell cultures: Chemical factories of secondary metabolites. **Biotech. Adv.** 20: 101-153.

Renaudin, J.P. 1989. Differen mechanisms control the vacuolar compartmentation of ajamalicine in *Catharanthus roseus* cell culrutes. **Plant Physiol. Biochem.** 27: 613-621.

Rout, G.R., S. Samantaray and P. Das. 2000. *In vitro* manipulation and propagation of medicinal plants. **Biotech. Adv.** 18: 91-120.

Rout, G.R., C. Saxena, S. Samantaray and P. Das. 1999a. Rapid plant regeneration from callus cultures of *Plumbago zeylanica*. **Plant Cell, Tiss. Org. Cult.** 56: 47-51.

Rout, G.R., C. Saxena, S. Samantaray and P. Das. 1999b. Rapid clonal regeneration of *Plumbago zeylanica* Linn. **Plant Growth Regul.** 28: 1-4.

Russin, W.A., D.D. Ellis, J.R. Gottwald, E.L. Zeldin, M. Brodhagen and A.F. Evert. 1995. Immunocytochemical localization of taxol in *Taxus cuspidate*. **Int. J. Plant Sci.** 156: 668-678.

Sáenz-Carbonell, L.A., I.E. Maldonado-Mendoza, O. Moreno-Valenzuela, R. Ciau-Uitz, M. López-Meyer, C. Oropeza and V.M. Loyola-Vargas. 1993. Effects of the medium pH on the release of secondary metabolites from roots of *Datura stramonium*, *Cartharanthus roseus* and *Tagetes patula* cultured *in vitro*. **Appl. Bioch. Biotech.** 38: 257-267.

Saito, K., H. Sudo, M. Yamazaki, M.K. Nakamura, M. Kitajima and H. Takayama. 2001. Feasible production of camptothecin by hairy root culture of *Ophiorrhriza pumila*. **Plant Cell Rep.** 20: 267-271.

Santiago, L.J.M., R.P. Louro and D.E.D. Oliveira. 2000. Compartmentation of phenolic compounds and phenylalanine ammonia-lyase in leaves of *Phyllanthus tenellus* Roxb. and their induction by copper sulphate. **Ann. Bot.** 86: 1023-1032.

Saralamp, P., W. Chuakul, R. Temsiririrkkul and T. Clayton. 1996. **Medicinal Plants in Thailand.Vol. I**. Amarin Printing and Plublishing Plublic Co., Ltd.

Saslowsky, D. and B.W. Shirley. 2001. Localization of flovonoid enzymes in *Arabidopsis* roots. **The Plant J.** 27: 1-37.

Satyanarayana, R. and G. T.Gujar. Bioactivity of naturally occurring plumbaginoids against *Dysdercus koenigii* Fabricus. **Shashpa** 6: 29-35. Cited S.R. Paiva de, M.R. Figueiredo, T.V. Aragão and M.A.C. Kaplan. 2003. Antimicrobial activity *in vitro* of plumbagin isolated from *Plumbago* species. **Mem. Inst. Oswaldo Curz.** 98: 959-961.

Satheesh Kumar, K. and K. V. Bhavanandan. 1988. Micropropagation of *Plumbago rosea* Linn. **Plant Cell, Tiss. Org. Cult.** 15: 275-278.

Schraut, D., C.I. Ullrich and W. Hartung. 2004. Lateral ABA transport in maize roots *(Zea mays*) : visualization by immunolocalization. **J. Expt. Bot.** 55: 1635-1641.

Selvakumar, V., P.R. Anbudurai and T. Balakumar. 2001. *In vitro* propagation of the medicinal plant *Plumbago zeylanica* L. through nodal explants. **In Vitro Cell Develop Biol.** 37: 280-285.

Sigma-Aldrich. 1999. Plumbagin. Available Source: https://www.sigma-aldrich.com

Singh, U.V. and N. Udupa. 1997. Reduced toxicity and enhanced antitumor efficacy of betacyclodextrin plumbagin inclusion complex in mice bearing Ehrlich ascites carcinoma. **Ind. J. Physiol. Pharmacol.** 41: 171-175.

Sirvent, T and D. Gibson. 2002. Induction of hypericins and hyperforin in *Hypercum perforatum* L. in response to biotic and chemical elicitors. **Physiol. Mol. Plant Pathol.** 60: 311-320.

Stensen, W. and E. Jensen. 1994. High-performance liquid chromatrographic separations of naphthoquinones and their derivatives. **J. Chromatogr.** 659: 87-93.

Swain, T. L. 1977. Secondary compounds as protective agents. **Annu. Rev. Plant Physiol.** 28: 479-485.

Tatreerod, S., P. Saralamp and S. Chanprame. 2003. Hairy root induction and culture of *Plumbago indica* L. pp. 306. *In* **The 3 rd World Congress on Medicinal and Aromatic plants for Human Welfare.** Ching Mai, Thailand.

Van Steenis, C.G.G.J. 1949. Plumbaginaceae. pp.107-112. *In* C.G.G.J., Van Steenis. ed., **Flora Malesiana.** Ser. 1, vol. 4. P. Noordhoff, Groningen.

Vander, P., K.m. Vårum, A. Domard, N.E.E. Gueddari and B.M. Moerschbacher. 1998. Comparison of the ability of partially N-acetylated chitosans and chitooligosaccharides to elicit resistance reactions in wheat leaves. **Plant Physiol.** 118: 1353-1359.

Vasconsuelo, A., A.M. Giulietti and R. Boland. 2004. Signal transduction events mediating chitosan stimulation of anthraquinone synthesis in *Rubia tinctorium*. **Plant Sci.** 166: 405-413.

Vasconsuelo, A., S. Morelli, G. Picotto, A.M. Guilietti and R. Boland. 2005. Intracellular calcium mobilization: A key step for chitosan-induced anthraquinone production in *Rubia tinctorium* L. **Plant Sci.** 169: 712-720.

Verma, P.C., D. Singh, L.U. Rahman, M.M. Gupta and S. Banerjee 2002. *In vitro* studies in *Plumbago zeylanica*: rapid micropropagation and establishment of higher plumbagin yielding hairy root cultures. **Plant Physiol.** 159: 547-552.

Villavicencio, M.A. and B.E. Perez-Escandon. 1992. Plumbagin activity (from *Plumbago pulchella* Boiss. Plumbaginaceae) as afeeding deterent for three species of Orthoptera. **Folia Ethanol. Mex.** 86: 191-198.

Walker, T.S., H.P. Bais and J. M. Vivanco. 2002. Jasmonic acid-induced hypericin production in cell suspension cultures of *Hypericum perforantum* L. (St. John's wort). **Phytochem.** 60: 289-293.

Wang, C., U. Jarlfors and D. F. Hildebrand. 1999. Regulation and subcellular localization of auxin-induced lipoxygenases. **Plant Sci.** 148: 147–153.

Watson, L., and M.J. Dallwitz. 1992. The Families of Flowering Plants: Descriptions, Illustrations, Identification, and Information Retrieval. Version: 19th August 1999. Available Source:http://biodiversity.uno.edu/delta/, Febuary 20, 2003.

Willims, R.R. 1995. The chemical microenvironment, pp. 405-439. *In* **Automation and Environmental Control in Plant Tissue Culture** Kluwer Academic Publishers. Dordrech. Cited A. Jacob and N. Malpathak. 2004. Green hairy root cultures of *Solanum khasianum* Clarke-a new route to *in vitro* solasodine production. **Current Sci.** 87: 1142-1147.

Wink, M. 1987. Production of secondary metabolites by plant cell cultures in relation to the site and mechanism of their accumulation, pp. 477- 484. *In* B.Matin**,** ed. **Plant Vacuoles**. Plenum Press, New York.

Yazaki, K., H. Matsioka, K. Shimomura, A. Bechthold and F. Sato. 2001. A novel dark-inducuble protein, LeDI-2, and its involvement in root-specific secondary metabolism in *Lithospermum erythrorhizon*. **Plant Physiol.** 125: 1831-1841.

Yu, F., D. Zhang, F. Bai and L. An. 2005. The accumulation of isocamptothecin A and B in cell suspension culture of *Camptotheca acuminate*. **Plant Cell, Tiss. Org. Cult.** 81: 159-163.

Yuan, Y.J., C. Li, Z.D. Hu, J.C. Wu and A.P. Zheng. 2002. Fungal elicitor-induced cell apoptosis in suspension cultures of *Taxus chinensis* var. *mairei* for taxal production. **Process. Biochem.** 30: 193-198.

Zárate, R. 1999. Tropane alkaloid production by *Agrobacterium rhizogenes* transformed hairy root cultures of *Atropa baetica* Willk. (Solanaceae). **Plant Cell Rep.** 18: 418-423.

Zavala, M.E. and D.L. Brandon. 1983. Localization of a phytohormone using immunocytochemistry**. J. Cell Biol.** 97: 1235-1239.

Zhao, J., L.C. Davis and R. Verpoorte. 2005. Elicitor signal transduction leading to production of plant secondary metabolites. **Biotech. Adv.** 23: 283-333.

Zhao, D.X., C.X. Fu, Y.S. Han and D.P. Lu. 2005. Effect of elicitation on jaceosidin and hispidulin production in cell suspension cultures of *Saussurea medusa*. **Process. Biochem.** 40: 739-745.

Zhao, D., Y. Huang, Z. Jin, W. Qu and D. Lu. 2003. Effect of aggregate size in cell cultures of *Saussurea medusa* on cell growth and jaceosidin production. **Plant Cell Rep.** 21: 1129-1133.

Zhao, J., W.H. Zhu and Q. Hu. 2001. Selection fungal elicitors to increase indole alkaloid accumulation in *Catharanthus roseus* suspension cell culture. **Enzyme Microb. Technol.** 28: 666-672.

Zheng, J. and B.D. Hammock. 1996. Development of polyclonal antibodies for detection of protein modification by 1,2-naphthoquinone. **Chem. Res. Taxicol.** 9: 904-909.

Zid, S.A. and Y. Orihara. 2005. Polyacetylenes accumulation in *Ambrosia maritime* hairy root and cell culrures after elicitation with methyl jasmanate. **Plant Cell, Tiss. Org. Cult.** 81: 65-75.

APPENDICES

Appendix Table 1 Composition of plant tissue culture media

Constituents	Concentration in culture medium (mg/l)		
	Murashige and Skoog (1962)	Gamborg B5 (1968)	MS-B5
Macro nutrients			
KNO_3	1900	2500	1900
NH_4NO_3	1650	-	1650
$(NH_4)_2SO_4$	-	134	-
$MgSO_4.7H_2O$	370	250	370
$CaCl_2.2H_2O$	440	150	440
KH_2PO_4	170	-	170
$NaH_2PO_4.H_2O$	-	150	-
Micro nutrients			
$MnSO_4.H_2O$	-	10	-
$MnSO_4.4H_2O$	22.3	-	22.3
KI	0.83	0.75	0.83
H_3BO_3	6.2	3.0	6.2
$ZnSO_4.7H_2O$	8.6	2.0	8.6
$CuSO_4.5H_2O$	0.025	0.025	0.025
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
$CoCl_2.6H_2O$	0.025	0.025	0.025
$FeSO_4.7H_2O$	27.8	27.8	27.8
Na_2EDTA	37.3	37.3	37.3
Organics			
Nicotinic acid	0.5	1.0	1.0
Pyridoxine-HCl	0.5	1.0	1.0
Thiamine-HCl	0.1	10.0	10.0
myo-Inositol	100	100	100
Glycine	2.0	-	-

Appendix Table 2 Composition of Czapex's Dox medium

Constituents	Concentration in culture medium (g/l)
$NaNO_3$	2
K_2HPO_4	1
$MgSO_4.7H_2O$	0.5
KCl	0.5
$FeSO_4.7H_2O$	0.01

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