CHAPTER 2

LITERATURE REVIEW

2.1 Delimitation and morphological characteristics of *Stemona* spp.

2.1.1 *Stemona curtisii* Hook. f

Twiner, glabrous; roots tuberous, in clusters, 5 – 10 cm long. Leaves alternate, elliptic-ovate, 5 – 15 x 5 – 12 cm, cordate or truncate at base, entire at margins, acuminate at apex, membranous, 7 – 13-veined at base; petioles 2 – 7 cm long. Raceme axillary, to 10 cm long; peduncle 5 – 8 cm long. Flowers caliber (c.) 2 cm across, greenish white; pedicels c. 5 mm long; bracts ovate, c. 6 x 1.5 mm, persistent. Tepals 4, lanceolate, c. 2.5 x 0.5 cm, rounded at base, entire at margins, acuminate at apex, greenish white with purple veins. Stamens 4, erect, purple; filaments 2 – 3 mm long; anthers linear-lanceoloid, c. 2.5 cm long, acuminate at apex, brown, 2-loculed. Ovary oblongoid, c. 1.5 x1 mm, 1-loculed; ovules 3 to many; style c. 1.5 mm long; stigma obtusely acute at apex, minutely papillose. Capsules ovoid, c. 3 x 1 cm, with persistent tepals; seeds few, c. 16 x 2.5 mm, acuminate at apex, arillate (Figure 2.1) (Marugun, 2010).

Location: Thailand (South-Western: Kanchanaburi, Ratchaburi, Phetchaburi, Prachuap Khiri Khan; Peninsular:Chumphon, SuratThani, Krabi, Nakhon Si Thammarat, Phatthalung, Trang, Satun, Songkhla, Pattani, Narathiwat), Sri Lanka, Sumatra (Lepar Archipelago), Peninsular Malaysia (type).

Ecology and Habitat: Deciduous, evergreen forest, near coastal areas, on riverbanks, near waterfalls, near brush and bushes; on sand, limestone, and granitic soil; from sea level to 600 m altitude. Flowering: December to July; producing fruit: February to November. (Inthachub *et al.* 2010)

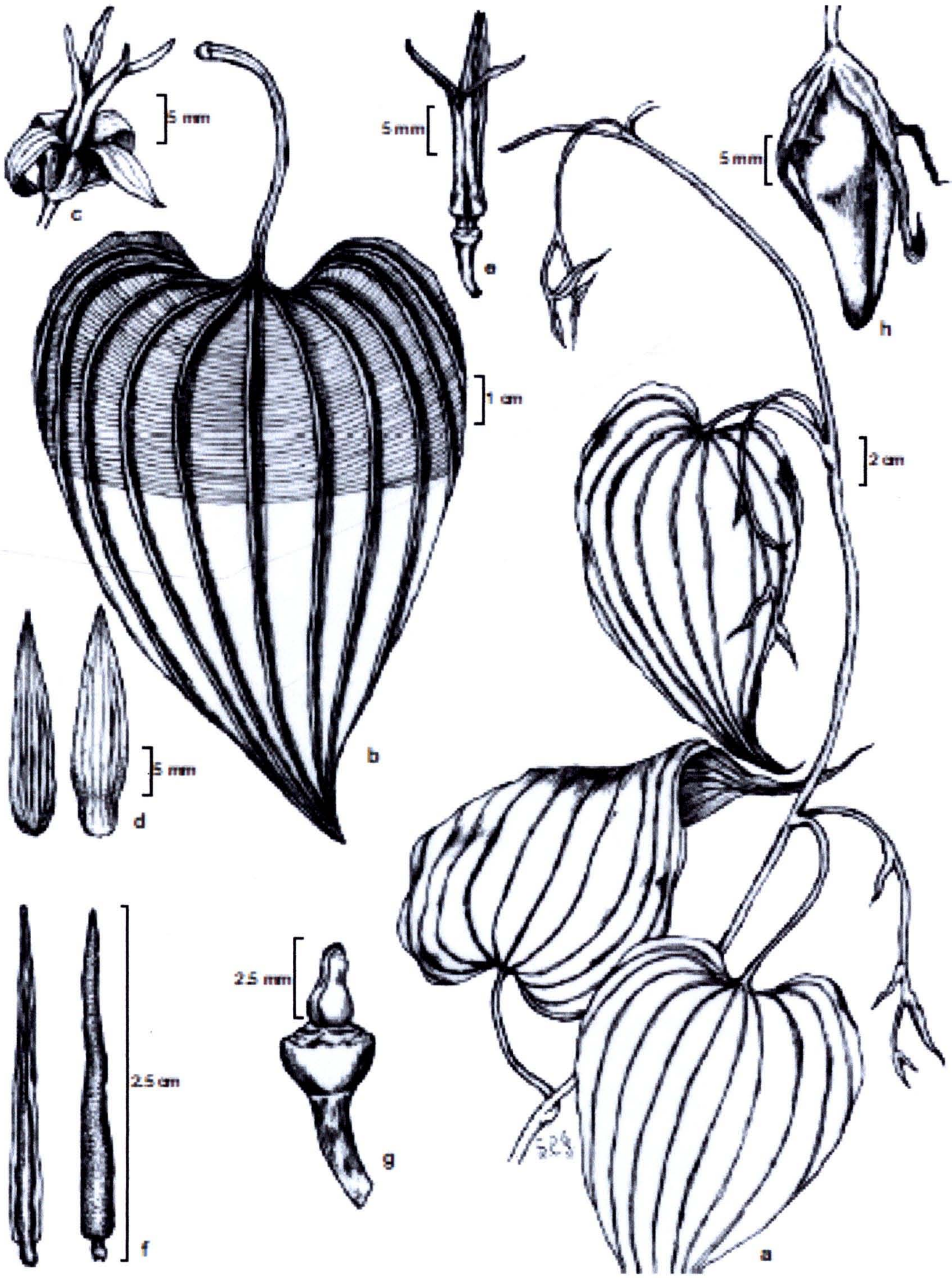
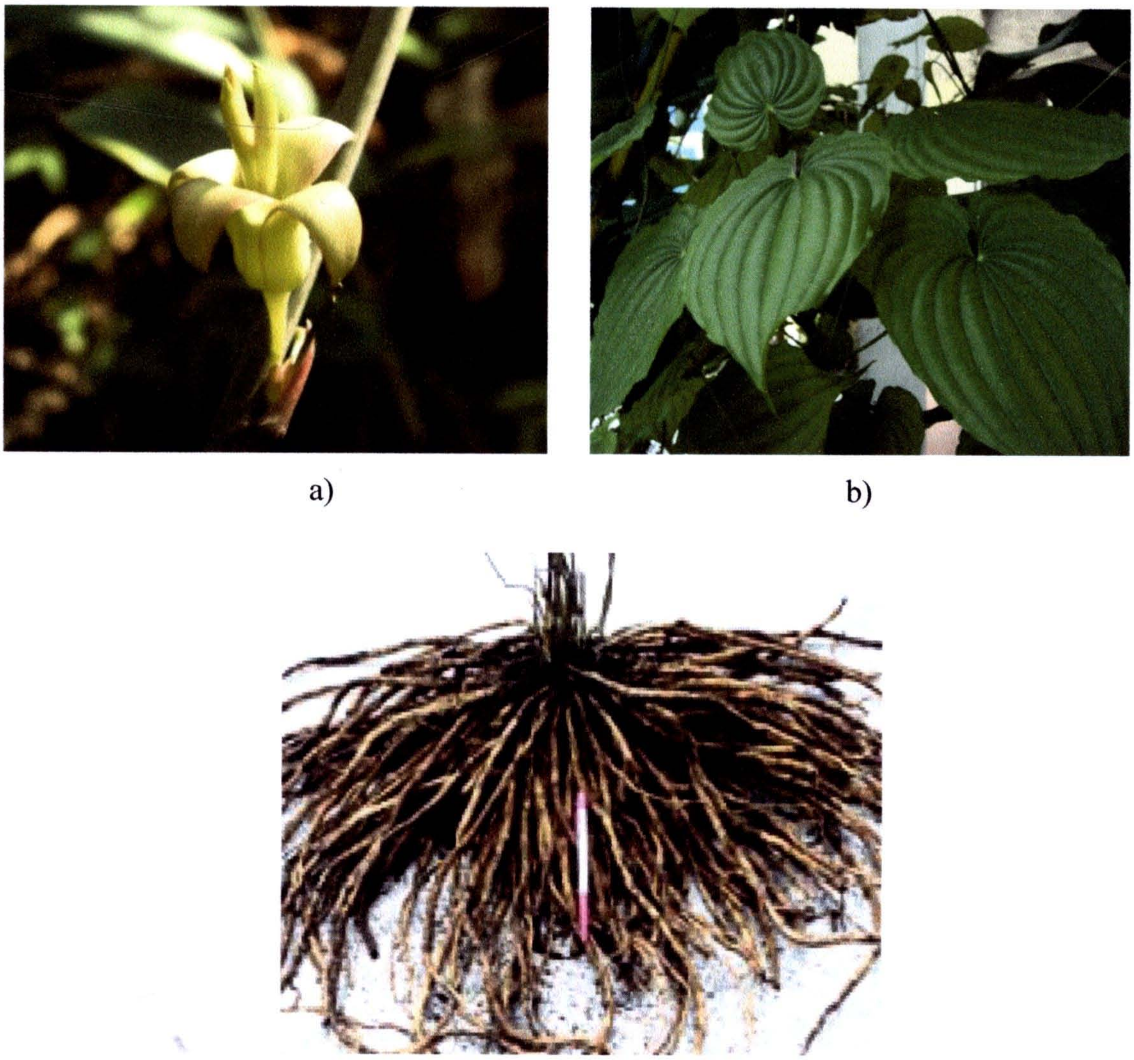


Figure 2.1 *Stemona curtisii* Hook f: a. Twig, b. Leaf, c. Flower, d. Tepals, e. Androecium, f. Stemens, g. Ovary with pedicel, h. Capsule with persistant tepals. (Marugun, 2010)

2.1.2 *Stemona* sp.

The intact plants of an unidentified *Stemona* species were found at Amphur Mae Moh, Lampang Province, Thailand, The plant material was identified by Mr. James Maxwell, and a voucher specimen was deposited at the Herbarium (number 25375) of the Department of Biololgy, Chiang Mai University. Morphological traits of the plant were as follows: deciduous, perenial vine; both sides of the petal pale light yellow-cream, becoming more light green with maturity; filament, septum crest, connectives light green; anther locules pale light tan. The supporting information has photographs of this plant material (Figure 2.2).



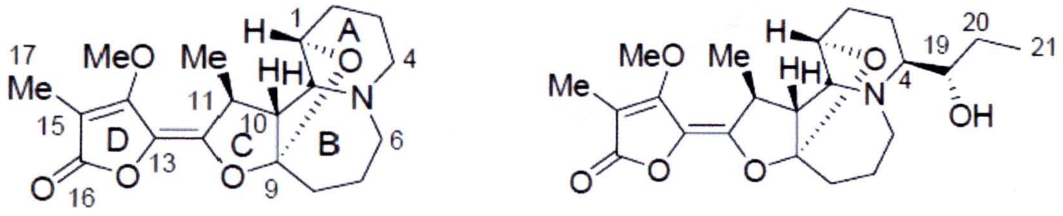
a) b)

c)

Figure 2.2 Unidentified *Stemona* sp. a) Flower b) Leaf and c) Tuberous root

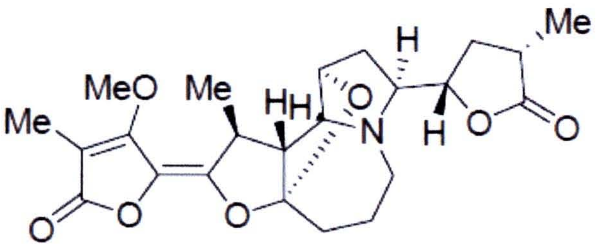
2.2 Bioactive of *Stemona* alkaloids

The isolation of stemocurtisine was reported and it was found to be the major alkaloid from the root extracts of *Stemona curtisii*, which was collected from the northern part of Trang Province in Thailand (Mungkornasawakul *et al*., 2003). In 2004, Mungkornasawakul *et al.* recorded the isolation of stemocurtisinol and oxyprotostemonine (Figure 2.3) from the same root extracts of *Stemona curtisii.*



a) Stemocurtisine

b) Stemocurtisinol



c) Oxyprotostemonine

Figure 2.3 Chemical structures of three alkaloids: a) stemocurtisine b) stemocurtisinol and c) oxyprotostemonine

The method recommended by the WHO was used to measure the larvicidal activity of the crude root extract of *S. curtisii* on mosquito larvae (*Anopheles minimus* HO) in order to determine the LC_{50}. A LC_{50} of 81 ppm was shown for the crude ethanol extract, while the individual alkaloid component displayed substantially higher potency (LC_{50} of 4-39 ppm). Oxyprotostemonine was found to be the most potent with a LC_{50} of 4 ppm (Mungkornasawakul *et al.*, 2003).

Roots of unidentified *Stemona* species from Lampang Province, Northern Thailand had been reported the isolation of 1′,2′-didehydrostemofoline (Figure 2.4) (Sastraruji *et al.*, 2005). Kaltenegger *et al.* (2003) demonstrated that 1',2'didehydrostemofoline had insecticidal activity against neonate larvae of *Spodoptera littoralis* with a LC 50 of 0.8 ppm. which caused hyperactivity of the larvae resulting in their sudden death and Baird *et al.* (2009) reported that 1′,2′-didehydrostemofoline has shown the highest inhibitory activity of acetylcholinesterase which indicated its potential for the treatment of Alzheimer's disease.

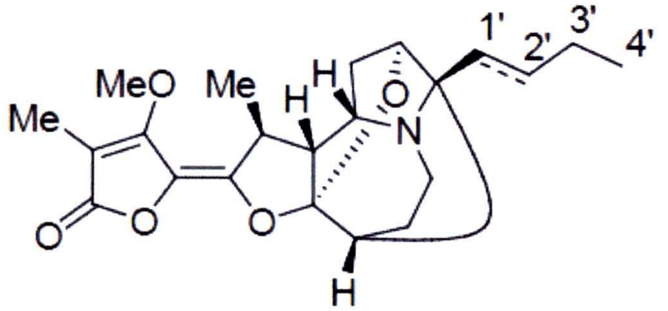


Figure 2.4 The chemical structure of 1′,2′-didehydrostemofoline (Pyne *et al.*, 2007)

2.3 Micropropagation of *Stemona* spp.

The induction of multiple shoots of *Stemona* spp. were investigated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), while various types of cytokinins (6-benzylaminopurine (BAP), benzyladenine (BA) and thidiazuron (TDZ)) and the roots were induced when the isolated shoot was transferred on MS medium supplemented with various auxins (indole-3-acetic acid (IAA) and naphthalene acetic (NAA)). The highest shoot multiplication rates of *Stemona curtisii* Hook f. were acquired by the induction from auxiliary bud explants on solid MS medium supplemented with 20 μM BAP. Then, the highest root formations were acquired by the induction of shoots and were cultured on MS medium and supplemented with 10 μM IAA (Montri *et al.*, 2006). Similarly, shoot tip cultures of *Stemona curtisii* Hook f. on the MS medium containing 4 mg/L BA and 1 mg/L NAA were induced using multiple shoots (3.33 shoots per explant) over a 4-week period. Rooting was achieved by the transference of the isolated shoot to MS medium with NAA (1 mg/L) (Chidburee *et al.*, 2008). New shoots were induced when shoot cultures of *Stemona tuberosa* Lour were put on MS medium supplemented with 1.0 mg/L TDZ, 5 and 10 mg/L BA. This resulted in 3.1±0.52, 2.62±0.12 and 2.6±0.76 shoots per explant, respectively (Singlaw *et al.*, 2008).

2.4 Plant tissue cultures and secondary product compounds (Lila, 2005).

Since the initial applications of *in vitro* technology, the ability of plant tissues, cells and organ cultures to acquire and maintain many of the valuable chemical compounds of the parent plant has been widely acknowledged. (Lila, 2005). The emerging demand for products that are both natural and sustainable has brought attention back to *in vitro* plant materials in the applications of phytochemical products. This trend has also contributed to an increase in research into the potential of *in vitro* secondary products.

It should be noted that the incentives for such research extend beyond the commercial aspects. Chemical products derived from intensely regulated *in vitro* culturing can contribute to a greater understanding of how the applications of these tissue cultures can be applied to both biochemical and metabolic processes.

The secondary compounds derived from plant tissues have been utilized in a broad variety of applications in both commercial and industrial processes. *In vitro* plant cultures of parent plants can produce high-value, natural products of significant potential, when generated via stringent scientific laboratory cultures. Both plants and plant cell cultures have proven to possess high potential as resources for a wide range of applications, such as in the way of enzymes, preservatives, flavorings, fragrances, natural pigments, cosmetics (known specifically as cosmeceuticals), both bio-based fuels and plastics, as well as other bioactive compounds.

There are distinct advantages to secondary products derived from plant cell cultures over the *in vivo* cultivation of the parent plant, which include:

- Simple, reliable and predictable method of production
- Efficient and effective isolation of phytochemicals
- *In vitro* cell culture compounds maintain the full integrity of the parent plant
- Cell cultures are not as susceptible to interfering compounds which commonly occur in the field
- Superior models of elicitation can be derived from cell cultures
- Cell cultures can be radio-labeled in the laboratory, therefore their impacts and influences can be traced metabolically when applied to secondary products
- Large volumes of defined and standardized phytochemicals can be obtained from cell cultures

The culturing of plant cells in the laboratory is not limited by the growing seasons that exist in the field and therefore can be produced throughout the year. This supports the claim that their production can be considered predictable, reliable and free from the influences and effects of environmental changes in weather. There have even been cases where the fresh (per gram) yield of the secondary products derived from cell cultures, have exceeded those yields that have been acquired from the same plant species in the field (Lila, 2005).

Additionally, laboratory cultivated plant cell cultures can be manipulated *in vitro* in order to eliminate any flavors or odors that may be thought of as unwanted or unpleasant. These plant cell cultures are also not subject to restrictions arising from geographic boundaries or political barriers. In the event that high-value products are derivatives of endangered plant species, *in vitro* plant cell cultures provide a functional alternative to the wild-grown, indigenous plants that may prove to be difficult to locate and collect. *In vitro* tissue extraction is also much less complicated than the extraction process that is required in order to extract complex tissues from the actual parent plant. In the secondary products that have been derived from plant cell tissues, we are provided with the unique opportunity to alter the chemical make-up of the resulting phytochemical product by tailoring its essential chemical profile in such a way that it would generate a product or compound of greater specific value to humans. This is often the case when a desired metabolite is only produced in specific plant tissues or glands of the original plant. If we consider ginseng (*Panax ginseng* C.A. Meyer), the metabolite saponin, for example, is produced exclusively in the ginseng roots and therefore it would require the cultivation of *in vitro* root cultures of the ginseng plant in order produce saponin. In the same way, the herbal plant commonly known as St John's Wort *(Hypericum perforatum)* accumulates its desirable hyperforins and hypericins in the foliar glands, but has not revealed the ability to produce the same phytochemicals in the undifferentiated cells (Smith *et al.*, 2002). Another example involves the tobacco plant (*Nicotiana tabacum* L.), where the conversion of lysine to anabasine occurs in its roots and is then followed by a secondary conversion of anabasine to nicotine in the leaves. The shoot cultures and callus of the tobacco plant itself can produce only minute amounts of nicotine because the specialized compound anabasine is absent in that part of the plant. There are also instances where a primary stage of conversion in a cell culture must initiate before the synthesizing of a final product can occur. This naturally occurring biosynthesis of a specialized process can be seen as a built-in defense trait that could be acknowledged to isolate a potentially toxic compound. There are three very well-known examples of commercially established products that are the result of *in vitro* cultivation. These are ginseng saponins, shikonin, and berberine, and each of them has been successfully cultivated and applied to medicinal applications. Of these, shikonine and berberine are the products of plant cell cultures, whereas ginseng has been widely cultivated from root cultures. The advancement of a variety of secondary *in vitro* products to the commercial market place is a result of an extensive push in the research of plant tissue cultures. A singular example of this would be how cell cultures contribute to the production of vanillin. While there is significant potential in the commercial industry for secondary product derivatives, there have been numerous cases where the secondary metabolite product of the *in vitro* process has not been commercially successful. The overall bio-process continues to be refined world-wide in an effort to develop new methods and techniques of secondary metabolite production. Extracts of the *in vitro* plant cell culture process can be found in a multitude of product categories that include flavorings such as peppermint, spearmint, vanilla, coffee, fruit and seaweed flavors; as well as spices and sweeteners; edible food colorings and medicinal treatments; non-edible pigments used in the cosmetic and textile industries; both fragrances and essential oils; and natural forms of insecticides and bioactive phytoalexins that have been successfully applied to pest management systems. Perhaps most notably, a great deal of research and development has been focused on the production of natural medicines and compounds from plant cell cultures that possess chemoprotective properties. In this group we can cite the phytochemical taxol in the treatment of ovarian cancer and ajmalicine (derived from *C. roseus*) for the treatment of circulatory problems (Lila, 2005).

2.5 Secondary metabolite production using root cultures

Plants depend on root systems as the plant organ that absorbs water and mineral nutrients from the soil or, in the case of hydroponics, other liquid medium. But plant roots are the recipients of sugars that are processed and delivered by the above ground segment of the plant. Thus, root cultures may be grown in a liquid medium, which contains minerals as well as organic nutrients. Under such conditions, when root systems are cut, they may continue to display an ability to grow in an elongated fashion but will not branch out or hold a sustainable level of growth. If phytohormones are introduced to instigate growth, the result will likely be a disorganized propagation culture. However, bio-technological techniques can be integrated into the cultivation of root cultures for the production of secondary metabolites. Advantages over intact parent plant derivatives include a shorter required period of time involved in growth and maturation, as well as a fairly significant potential for greater yields (Bunrathep, 2006). For example, shoot apices and lateral bud cultures of *Stemona collinsae* Craib in solidified MS medium containing 2 mg/L BA and 0.1 mg/L NAA were investigated. Samples of 500 g fresh-weight plant material were extracted in alcohol and then analysed by liquid -liquid chromatography and high performance liquid chromatography (HPLC) for the identification of stemofoline and 16,17-didehydro-16(*E*)-stemofoline. Chromatogram of the extraction from *Stemona* tissue culture showed a similar pattern of stemofoline and 16,17-didehydro-16(*E*)-stemofoline to *Stemona* root extract from naturally grown plants. Although a comparative study on chromatogram patterns of *Stemona* plantlets cultured under light and dark conditions and revealed that the production of stemofoline and 16,17-didehydro-16(*E*)-stemofoline synthesis could very well be induced by light (Burikam *et al.*, 2005).

2.6 Secondary metabolite production enhancement through elicitation

The promotion of the secondary metabolism in plant cell and tissue cultures by the use of elicitors has become routine. Due to the fact that plant secondary metabolites are active participants in inducible defense mechanisms, strategies for a number of species have been developed that would imitate the stimulus that initiates their accumulation. These include the use of the crude cell debris of heat-killed fungi or bacterial cultures, cell wall fractions from these microorganisms, or polysaccharides, mechanical wounding, and the impacts of different physicochemical stresses, such as heavy metals, salts or a low osmotic potential. All of this is due to the exposure of cultures to the chemical mediators involved in the response to these stimuli, such as methyl jasmonate (MJ) and salicylic acid (SA) (Vazquez-Flota *et al.*, 2009).

Methyl jasmonate (MJ)

The accumulation of saponin was enhanced by MJ in the adventitious roots of ginseng, and peaked after 7 days of culturing (Kim *et al.* 2003). The synergistic use of indole-3-butyric acid (25 µmol/L) and MJ (100 µmol/L) increased the ginsenoside production in the adventitious roots (Kim *et al.*, 2007). The usefulness of organic germanium as an elicitor was demonstrated when the addition of 60 mg/L organic germanium to ginseng adventitious root cultures and enhanced both the biomass and ginsenosides accumulation (Yu *et al.*, 2006). Ajmalicine and catharanthine accumulation in *in vitro* cell suspension in the hairy roots and rootless shoot cultures of *Catharanthus roseus* were promoted by MJ treatments (Vazquez-Flota *et al.*, 2009).

The effects were observed after MJ was added externally (Ruiz-May *et al.*, 2009). Following the elicitation of *Catharanthus roseus* hairy roots with different concentrations of MJ, accumulation of alkaloids, such as ajmalicine, serpentine, ajmaline and catharanthine, had occurred and the changes were observed. In addition to the increased accumulation of alkaloids in the tissues, the root exudation of phytochemicals also increased in comparison to the non-treated hairy roots (control). Additionally, the secretion of several C. *roseus* hairy root metabolites was found to be induced by MJ.

The sesquiterpene lactone content and biomass accumulation were studied in the hairy root culture of *Cichorium intybus* for the effects of MJ. The guaianolidescrepidiaside B, 8-deoxylactucin, and the germacranolide sonchuside A were quantified by RP-HPLC. The growth of the hairy root culture was found to be affected by MJ. After 72 hours, jasmonate up-regulated the biosynthesis of the analysed sesqiterpene lactones in the culture. SA caused a transient increase in sonchuside A accumulation in the roots, by twice the level of the control, and a decrease of guaianolide content were caused by SA. (Malarz *et al*., 2007).

Anthocyanin producing by cell cultures of ohelo *(Vaccinium pahalae)* was elicited by MJ. The addition of 0.5 ppm MJ alone resulted in a 2-3 times increase in anthocyanin production over the control; therefore no additive effect on anthocyanin production was observed in any treatments combining MJ and 3-glucan, or FeEDDHA. Conditioning of the cells with a preculture in either MJ, 3-glucan, or FeEDDHA did not enhance anthocyanin production. MJ or 6-glucan with ibuprofen was used to elicit the cultures. This was because ibuprofen is a known inhibitor of jasmonate biosynthesis, and it will significantly stimulate anthocyanin production, while not inhibiting it. Therefore, this results in levels of accumulation in excess of any of the tested elicitor combinations. Yimin Fang and Pepin (1999) discussed the hypotheses for the observed influence of ibuprofen in this system.

The effects of the elicitors MJ on the production of bilobalide (B), ginkgolide A (GA), and ginkgolide B (GB) were studied in cell suspension cultures of *Ginkgo biloba* in order to increase productivity. It was found that MJ treatments increased the amounts of B, GA, and GB, concomitant with a minute decrease in cell growth. Following the treatment of 0.01mM MJ, GA and GB levels increased by 4.3 and 8.2 times over the controls at 12 h, but declined after 24 h. A maximum release of B after 12 h of exposure was produced by 1.0 mM MJ treatment and also increased the concentration of B in the culture medium up to 6.25 times the amounts of the controls. GA and GB production were transiently enhanced by the treatment of 1.0 mM of SA by up to 3.1 and 6.1 times, respectively, in comparison to the control (Seung-Mi *et al.*, 2006).

A significant interaction was observed between MJ and both indole-3-acetic acid (IAA) and 6-benzylaminopurine (BA) in order to influence the cell growth of cultured *Onosma paniculatum* cells. Increasing concentrations of MJ from 0.004–4.45 mM with or without IAA and BA resulted in a decrease in cell growth. However, the same concentrations of MJ (0–4.45 mM) increased the cell growth with IAA and BA, when added to the cultured cells in M9 medium, and shikonin production was found to be enhanced. Enhanced shikonin formation was achieved at 4 d after cell inoculation with the addition of MJ in the M9 medium, while the formation of shikonin was affected rather significantly by both the MJ/IAA and MJ/BA combinations. Additionally, shikonin content was enhanced when MJ concentrations were increased with IAA concentrations in the range of 0–28 mM and with BA concentrations in the range of 0–44.38 mM in the MJ/IAA and MJ/BA experiments, respectively. The optimal concentrations instead of combination of MJ and IAA were 4.45 mM and 0.28 mM, while MJ and BA concentrations of 4.45 mM and 2.22 mM were found to be the optimal combination for the shikonin formation. This result also showed that MJ increased phenylalanine ammonia-lyase (PAL) and p-hydroxybenzoic acid-geranyltransferase (PHB-geranyltransferase) activities during shikonin formation, but did decrease the activity of PHB-O-glucosyltransferase within 9 d after inoculation. These results also suggest that the regulation of the key enzyme activities will enhance shikonin formation in cultured *Onosma paniculatum* cells induced by MJ (Jian *et al.*, 2004).

Anthocyanin production in the callus cultures of *Daucus carota* were found to be stimulated by MJ. The highest levels of anthocyanin (0.37%) were observed in the cultures treated with 0.01 µM MJ. A higher activity of Ca^{2+}ATPase was observed in the MJ treatments, which suggested that the enhancement of anthocyanin by MJ could be mediated through the involvement of the calcium channel (Sudha and Ravishankar, 2003).

Transformed root cultures known as hairy root cultures have been shown to be good models for the study of many secondary metabolites. However, the compounds asiaticoside and madecassoside are only produced in insignificant amounts in the root of *Centella asiatica* (L.) Urban. Therefore, C. *asiatica* was transformed using *Agrobacterium rhizogenes* strain R1000 harboring pCAMBIA1302 encoded with the hygromycin phosphotransferase (hpt) with green fluorescence protein (mgfp5) genes while the hairy culture was then coupled with the elicitation technique. A frequency of up to 14.1% from a tissue junction between the leaf and petiole was used to obtain the hairy roots. After 7 days, abundant hairy roots were observed when co-cultivation of the plant with A. *rhizogenes* was implemented (36.1%). Transformation was confirmed by a polymerase chain reaction (PCR) and Southern blot analyses. No asiaticoside was detected in the hairy root samples after five weeks of inoculation. However, when 0.1 mM MJ was introduced as an elicitor to the culture medium for 3 weeks, a large amount of asiaticoside was produced (7.12 mg/g dry wt). In terms of gene expression, 12 h after MJ treatment the expression of the CabAS (C. asiatica putativeb-amyrinsynthase) gene in the hairy roots was significantly different from that of the control while this level was maintained for 14 days. Thus, the production of C. *asiatica* hairy roots could be optimized and the resulting cultures could be elicited with MJ treatment for the enhanced production of asiaticoside (Qk-Tae *et al*., 2007).

Six days of growth was found to be the optimal growth stage for enhancing ajmalicine production in *Catharanthus roseus* cultures with MJ. While, MJ added at 10 or 100 μM on day 6 was found to give a maximum ajmalicine production of 10.2 mg l−1, which was a 300% increase over that of non-elicited cultures (Lee-Pasons *et al.* 2004).

Ginsenoside production was enhanced in both the shake-flask (250 ml) and airlift bioreactor (ALR; 1 l working volume) with a single addition of 200 μM MJ to high-density cell cultures of *Panax notoginseng*. Elicitation was repeated in two additions of 200 μM MJ during cultivation and this further induced the ginsenoside biosynthesis in both cultivation vessels. Ginsenoside content of Rg1, Re, Rb1 and Rd in the ALR increased respectively, from 0.18±0.01, 0.21±0.01, 0.21±0.02 and 0 mgper100 mg dry cell weight (DW) in untreated cell cultures (control) to 0.32±0.02, 0.36±0.02, 0.72±0.06 and 0.08±0.01 mg per100 mg DW with the addition of MJ, and further increases of 0.43±0.02, 0.46±0.03, 1.09±0.07and 0.14±0.02 mg per100 mg DW were achieved with two additions of MJA. However, the activity of the Rb1 biosynthetic enzyme (UDPG-ginsenoside Rd glucosyltransferase) was also increased with a single elicitation by MJA and this increased again with repeated elicitation. This coincided well with the trend in the increase in Rb1 content. A strategy of MJ repeated elicitation combined with the feeding of sucrose was adopted, in order to further improve the overall cell density and ginsenoside production. The final cell density and total ginsenoside content in the ALR reached 27.3±1.5 g/l and2.02±0.06 mg per100 mg DW; and the maximum production of ginsenoside Rg1, Re, Rb1 and Rd was 111.8±4.7, 117.2±4.6, 290.2±5.1 and 32.7±8.1 mg/L, respectively. The results of this study will contribute to further refine the production of bioactive ginsenosides by plant cell cultures on a large scale (Wei *et al.*, 2005).

After inoculating the hairy root cultures of *Panax ginseng* with *Agrobacterium rhizogenes* KTTC 2703, they were further cultured in phytohormone-free MS liquid medium containing different concentrations of JA. Total ginsenoside production in ginseng hairy roots was enhanced with the addition of JA in the range of 1.0±5.0 mg l21 (4.8±23.8 mM). However, JA also strongly inhibited ginseng hairy root growth (Kee-Won *et al.* 2000).

Cell suspensions and hairy root cultures were established from shoot cultures of wild-harvested *Ajuga turkestanica* in order to develop a sustainable source of metabolism-enhancing phytoecdysteroids. The elicitor, MJ, was added to subculture media to increase phytoecdysteroid accumulation. In cell suspension cultures, 20-hydroxyecdysone (20E) content increased by 2 or 3 times with the addition of 125 or 250 µM MJ, respectively, when compared to unelicited cultures (Cheng *et al.*, 2008).

Root cultures of *Brugmansia suaveolens* were cultured by the infection of root tips with *Agrobacterium rhizogenes*. Successful transformation was done by the analysis of rol C and vir C genes using a PCR. Root cultures were studied for the formation of tropane alkaloids, such as hyoscyamine. The transformed cultures were incubated with potential elicitors, such as MJ, quercetin and SA, and then the biosynthesis of tropane alkaloids was stimulated. The profile and amounts of tropane alkaloids were analysed using capillary GLC-MS. At least 18 different tropane alkaloids were identified. The alkaloid accumulation was increased from the treatment of the cultures with 200 µM MJ by 25 times up to a level of 1 mg/g fresh weight, when compared to untreated controls. Quercetin enhanced the alkaloid production by 10 times (0.4 mg/g fresh weight) over a period of 24 h. Whereas 100 µM of SA decreased alkaloids to a level of 1 µg/g fresh weight (Zayedb and Winka, 2004).

Salicylic acid (SA)

It has been reported that SA has the ability to enhance the production of azadirachtin in the hairy roots of *Azadirachta indica* (Satdive *et al*., 2007), in a similar way as the feeding of 2 mM SA has been shown to stimulate tropane alkaloids by *Atropa belladonna* (belladonna) hairy root (Lee *et al*., 2001). The treatment of hairy root cultures of *Coleus forskohlii* with 0.1 mM SA was stimulated to a yield of 5.5 mg (2.0 more times than the control) rosmarinic production (Lee *et al*., 2001).

Total tanshinone (cryptotanshinone andtashinone IIA) contents in the cultures were 12.23 mg/L and 15.07 mg/L for the yeast elicitor (4 g/L), and the yeast elicitor plus 200 µmol/L SA treated cultures, respectively, when Ti transformed *Salvia mitiorrhiza* cells were cultured in a MS-NH_4 medium (MS without ammonium nitrate, containing 30 g/L of sucrose) at 25 °C in darkness for 18 d. However, only minute amounts of tanshinone were detected in the control or SA treated cells. In order to confirm these results, endogenous phytohormones were determined using ELISA kits. It was found that the ABA and iPAs contents in the yeast elicitor plus SA treated cell cultures did increased by 2.8 to 9.8 times and 3.6 to 5.8 times, respectively, while GA1 and IAA contents were decreased by 13.2%–56.9% and 34.8%–74.6%, respectively. Thus, higher levels of ABA and iPAs when combined with lower levels of GA1 and IAA will inhibit the growth of cells, and likely stimulate tanshinone production (Guo-Jing *et al*., 2003).

The effects of the elicitor SA on the production of bilobalide (B), ginkgolide A (GA), and ginkgolide B (GB) were studied in the cell suspension cultures of *Ginkgo biloba*, in order to increase productivity. MJ treatments increased the amounts of B, GA, and GB, concomitant with a slight decrease in cell growth. The level of B in the cells was increased 5.4 times over the controls at 12 h, but declined after 24 h when treated with 0.01 mM SA (Seung-Mi *et al.*, 2006).

The effects of SA on anthocyanin accumulation, endogenous titres of polyamines, as well as its effect on the ethylene production in callus cultures of *Daucus carota* were considered. The anthocyanin production in the callus cultures was found to be stimulated by SA. The highest levels of anthocyanin were recorded in the cultures treated with 200 μM SA0.36 %. Higher activity of Ca^{2+}ATPase was also found as a result of the SA treatments indicating that the enhancement of anthocyanin by SA could be mediated by the involvement of the calcium channel. Slightly higher titres of endogenous polyamines (Pas) were found to be a consequence of the treatment of the callus cultures with SA. (Sudha and Ravishankar, 2003).

SA as the signaling molecule was studied relative to the isoflavone accumulation in the hairy root cultures of *Psoralea corylifolia* L.1.55% dry wt of daidzein and 0.19% dry wt of genistein accumulated in the untreated hairy roots, which were used as a control. After two days of elicitation, 1 mM of SA concentration stimulated the maximum accumulation of daidzein (2.2% dry wt) and genistein (0.228% dry wt) (Shinde *et al.*, 2009).

The influence of SA as an elicitor on the growth and production of the tropane alkaloids; hyoscyamine and scopolamine were studied by observing leaf-derived root cultures of *Datura metel* L. that were established on B5 Medium containing1.2 μM IAA. However, the hyoscyamine and scopolamine contents in the control root cultures were 1.39 mg/g dw and 0.069 mg/g dw, respectively. The highest levels of hyoscyamine (4.35 mg/g dw) and scopolamine (0.28 mg/g dw) accumulation were obtained in cultures treated with 500 μM SA (Ajungla *et al.*, 2009).

Chitosan

The effects of elicitation on the colorant production were studied in order to increase the production of anthraquinone colorants in madder (*Rubia akane* Nakai) cell cultures. Chitosan was found to be the best biotic elicitor from nine plant-derived and microbe-derived polysaccharides. The total production in a 7-day culture was increased by about two times when elicited with 25 mg/L of chitosan in a seven-day culture, when compared to that of the unelicited cells. Anthraquinone production was increased over 3 days and maximum anthraquinone colorants were obtained within a 3-day treatment of chitosan. During the chitosan elicitation, the total production was increased by 1.3 times in the MS medium containing galactose, as compared to the medium containing sucrose. The production of anthraquinone colorants was not affected by the degree of deacetylation in chitosan and either the use of a growth regulator or the addition of a precursor. The anthraquinone concentration and specific anthraquinone content were increased by 1.3 times (0.69 g/L) and by 2.2 times (0.32 g/g DCW), respectively when madder cells were elicited at optimum conditions (Jin *et al.* 1999).

Indirubin production by *Polygonum tinctorium cells,* that were cultured in flasks during shaking, was investigated for the effects of chitosan. The optimum concentration of chitosan was found to be 200 mg/L, as well as the specific indirubin concentration (indirubin concentration per unit weight of cells) of 5.17 mg/g dry cell weight (DCW) were found to be the optimal conditions for indirubin production. In the presence of chitosan, the optimum period of elicitation was found to be 5 d. However, indirubin production was enhanced by 72% when the cells were cultured in the presence of 200 mg/L of chitosan for 5 d using SH medium supplemented with 2.72 mM calcium chloride and 5 mM indole (Ju Hwan, 1997).

A significant enhancement of anthocyanin accumulation was found to be the result of the elicitation of anthocyanin-producing cells of ohelo *(Vaccinum pahalae)* by biotic (purified 13-glucan and chitosan) elicitor. Whereas, anthocyanin production increased up to 1.9 and 1.6 times in the presence of biotic elicitors (10 mg/L) 3-glucan and 100 mg/L chitosan, respectively) (Yimin Fang and Pepin, 1999).

Isoflavone accumulation in the hairy root cultures of *Psoralea corylifolia* L was studied for the effect of chitosan. Untreated hairy roots (control) accumulated 1.55% dry wt of daidzein and 0.19% dry wt of genistein. Chitosan (2 mg/L) was found to be the most efficient elicitor to induce daidzein (2.78% dry wt) and genistein (0.279% dry wt) levels in the hairy roots was the untreated hairy root culture (as control), which accumulated 1.55% dry wt of daidzein and 0.19% dry weight of genisten (Shinde *et al.*, 2009).

Leaf explants were infected with *Agrobacterium rhizogenes* A4 in order to establish the transformation of root cultures of *Polygonum tinctorium* Lour, and these cultures were examined for their growth and indigo content under various culture conditions. SH medium revealed the highest yield for overall root growth (28 mg dry wt/30 ml) and indigo production (152 µg/dry wt) among the four different culture media that were tested. In SH medium, the best conditions for indigo production were found to be 30 g of sucrose l^{-1}, 2500 mgKNO_3 l^{-1}, 300 mg$NH_4H_2PO_4$ l^{-1}at pH 5.7. The addition of 200 mg/L of chitosan (186 µg/dry wt) and 20 U/L pectinase (181 µg/dry wt) caused the production of indigo in hairy roots to slightly increase (Young-Am *et al.*, 2000).

The production and release of hyoscyamine and scopolaminein hairy root cultures of *Brugmansia candida* were studied for the effects of chitosan, acetic acid and citric acid. Different concentrations and different media pH values of chitosan and acetic acid were tested. At a pH of 5.5 and at specific concentrations, acetic acid and chitosan increased the content of root scopolamine and hyoscyamine, and promoted the release of both alkaloids (Pitta-Alvarez and Giulietti, 1999).

After being applied to modified MS medium, chitosan in the range of 0.5 to 7.0 mg per g fresh weight callus was found to elicit suspended callus cultures of *Citrus grandis*, based on measurements of the conductivity of the culture medium. It was found that low concentrations of chitosan (0.5 mg/g fr. wt.) stimulate limonene production and at the same time increase linalool content. Cultures elicited with 1.0 mg chitosan/g fr. wt. callus and incubated for 2 hours were used to determine the maximum limonene and linalool accumulations. Limonene and linalool accumulation were successfully influenced by chitosan in a short period of time, but were found not to contribute to the permeabilization of the cells (Rahman *et al.*, 2003).

Yeast extract

After 6 days of elicitation, MJ, 50 µM, 0.5 mg yeast extract/l and 100 mg/L chitosan stimulated plumbagin production in *Drosera burmanii* whole plant cultures. While the yeast extract (0.5 mg/L) displayed the most efficient potential to enhance plumbagin production in the roots of D. *burmanii* to 8.8 ± 0.5 mg/g dry wt and was 3.5-fold higher than the control plants (Putalun *et al*., 2010).

The influence of yeast extract elicitor on the growth and production of the significant tropane alkaloids hyoscyamine and scopolamine were studied by employing the leaf-derived root cultures of *Datura metel* L., established on B5 medium containing 1.2 μM IAA. The total hyoscyamine and scopolamine contents in the control root cultures were 1.39 mg/g dw and 0.069 mg/g dw, respectively. Cultures treated with 0.75 g/L yeast extract were used to obtain the hyoscyamine (3.17 mg/g dw) and scopolamine (0.16 mg/g dw) accumulations (Ajungla *et al*., 2009).

2.7 Enhanced secondary metabolite production through precursor feeding

Several organic compounds have been added to the culture medium in order to enhance the synthesis of secondary metabolites within the biosynthetic pathways. The yield of the desired product may be increased by the exogenous supply of a biosynthetic precursor to the culture medium. The yield of the final product will likely be increased by the presence of an intermediate compound in or at the beginning of a secondary metabolite biosynthetic route. Supplying a precursor or intermediate compound will induce or increase the production of the plant secondary metabolites. This theory has been proven to be effective in many cases.

Sodium acetate

A 60-fold induction and secretion of *trans*-resveratrol into the medium has been achieved by the addition of sodium acetate to the hairy root cultures of peanuts (*Arachis hypogaea*) (Medina-Bolivar *et al*., 2007). The addition of 50 mg/L sodium acetate to callus cultures of *Artemisia annua*, on the 10th d produced 32.2 mg/L of artemisinin (Baldi and Dixit, 2008). The addition of 10 mg/L sodium acetate to the cell cultures of *Azadirachta indica* on the third day of culturing also showed a significant increase in the bioproduction of azadirachtin (Balaji, 2003).

Cell suspension and hairy root cultures were established from shoot cultures of wild-harvested *Ajuga turkestanica,* in order to develop a sustainable source of metabolism-enhancing phytoecdysteroids. Phytoecdysteroid accumulation did increase by adding precursors of phytoecdysteroids (acetate, mevalonic acid cholesterol) to the subculture media. In the hairy root cultures, phytoecdysteroid content was increased by the addition of sodium acetate, mevalonic acid, and MJ, but not cholesterol, in comparison to the unelicited cultures. 20-hydroxyecdysone content was increased by approximately 2 times to 19.9, 20.4 or 21.7 µg mg-1, respectively, by treating the hairy root cultures with 150 mg/L sodium acetate, or 15 or 150 mg/L mevalonic acid, when compared to the control (10.5 µg/mg) (Cheng *et al*., 2008).

Sucrose

Granular tissues isolated from a culture line of callus were used to establish the root cultures of *Duboisia myoporoides* and D. *Leichhardtii*. The granular tissues easily differentiated the roots, which effectively grew in liquid MS medium supplemented with indole-3-butyric acid (IBA) (2mg/L) and gibberellic acid (1mg/L). Atropine, scopolamine and nicotine were found to be produced by these cultured roots. Additionally, anabasine and nornicotine were detected in root cultures of D. *myoporoides*, and apoatropine was detected in the root cultures of D. *Leichhardtii*. Root growth and tropane alkaloid production were improved by a high concentration (7-10 % (w/v)) of sucrose in the medium (Kitamura *et al*., 1991).

In response to changes in the sucrose content (2-8%) of the culture media, saikosaponin productivity was examined in *Bupleurum falcatum* L. BFHR2 hairy root culture. It was found that the conditions for optimal root growth differed from the maximal saikosaponin production. The highest saikosaponin yield was achieved at 8% sucrose (Ahn *et al*., 2006).

Tyrosine

Agrobacterium rhizogenes ATCC15834 was used to obtain a culture of *Stephania suberosa*. The production of dicentrine was recorded as 8.92 ± 0.07 mg/g dry wt on day 35 of the culture. The growth and production of *S. suberosa* was investigated for the effects of sucrose content, tyrosine, and medium strength. The optimum content for the growth and dicentrine accumulation in *S. suberosa*hairy roots was reported to be at 6% (w/v) sucrose. Dicentrine production was enhanced by the utilization of a precursor from the tyrosine feeding. At day 40 of the culture, the medium with 1.0 mM had the highest effect on dicentrine accumulation in the hairy roots [(14.73 ± 0.47) mg/g dry wt]. A solution of MS medium at 25% was suitable for biomass and dicentrine production in the hairy roots and had a significant potential to produce dicentrine from the hairy roots of *S. suberosa* (Putalum *et al.*, 2009).

Alkaloid production by *C. peruvianus* plants compared with callous tissues, revealed that alkaloid levels were almost double in the callous tissues as they were of those found to be present in the shoots of the *C. peruvianus* plants. The ratio of alkaloid concentration between the mature plants and the morphologically undifferentiated cells of the callous tissue was 1:1.7. It was also observed that there was a relationship between the culture medium containing tyrosine and alkaloid production in the callous tissues of *C. peruvianus*. Additional factors, such as the presence of tyrosine, increased levels of tyrosine or other conditions of the culture medium, may increase alkaloid production and should formally be considered factors for inducing higher alkaloid production in C. *peruvianus* callus tissues (De Oliveira and Da Silva Machado, 2002).

2.8 Enhanced secondary metabolite production through culture conditions

The development of the improved instrumentation for the cultivation of plant cells is a secondary goal of this research. For example, in order to permit light to reach plant cells during division and product accumulation, the specific design of bioreactors have been improved. In a significant number of cases, the successful synthesis and maximal expression of secondary metabolites is dependent on the infiltration of light at optimum intensities and spectral qualities. In recent years, enhanced plant product yields have also been reached by modifications in aeration, agitation, pH of medium cultures, temperature and the supply of nutrients.

Culture temperature

The effects of different temperatures on cell growth and hyocyamin production in the cell cultures of *Datura stramonium* have been previously reported (Iranbakhsh *et al.*, 2007). Additionally, the effects of temperature on alkaloid production showed that the maximum dosage of alkaloid production was at 20 °C with the highest fresh and dry weight of biomass at 25 °C. This is similar to what Rhoton and Bouteraouy (1994) reported, where hyocyamin in tissue culture at 20 or 25 °C did increase as compared with tissue culture at 30 °C in *Datura stramonium.*

pH of the medium

Culture of *Panax ginseng* C. A. Meyer were investigated under various pH values, as well as various concentrations of sucrose, nitrogen and phosphate and elicitors in order to determine root growth and saponin production. Root growth was not significantly affected by the pH of the medium, but the saponin content was affected. The maximum saponin content of 0.26 % was obtained at pH 6.0, whereas the optimal concentrations of sucrose, nitrogen and phosphate on root growth and saponin production were 30 g/l, 382.7 mg/L and 40.40 mg/L, respectively (Kim *et al.*, 2005).

The infection of the *Agrobacterium rhizogenes* strain ATCC 15834 into the leaf explants was used to establish the hairy roots of *Plumbago indica* at a high frequency of 90%. Darkness in hormone-free liquid MS medium containing 3% sucrose was used to establish axenic root cultures. The highest plumbagin content was found to have accumulated in the roots at their most established level of growth. It should also be indicated that a low pH (4.6) and a low concentration of sucrose (1 %) were beneficial for root growth in darkness, while pH 5.6 and 3 % sucrose under continuous irradiance, enhanced plumbagin accumulation in the roots at up to 7.8 mg g-1(d.m.). Continuous irradiance was used to achieve direct shoot regeneration from the hairy root culture, this, therefore, indicated that it is an effective way of obtaining transformed *P. indica* plants (Gangopadhyay *et al.*, 2008).