CHAPTER 2
LITERATURE REVIEW

2.1 General characteristics of Plookao

Plookao is an ethnobotanic species of northern Thailand and countries in Southeast Asia. It is belonged to the family Saururaceae. In Asia, this herb has been separated into two distinct chemotypes, but there is only one distinct chemotypes in Thailand that is *Houttuynia cordata* Thunb. The plant has strong smell like fishwort. (Medicinal Plant Research Institute, 2003).

General characteristic of the plant in the family Saururaceae is a perennial annual crops. *Houttuynia cordata* Thunb. is an herbaceous perennial ground cover plant. The height of the plant is between 20 and 80 cm. Proximal part of the stem is trailing with adventitious roots (Figure 2.1). While the distal part of the stem grows vertically. The leaves are alternate, broadly heart-shaped with 4-9 cm. long and 3-8 cm. broad attached to the petiole. Flowers are greenish-yellow, borne on a terminal spike 2-3 cm. long with 4-6 large white basal bracts. The plant shows inflorescence with bract at the base and with a large bouquet of flowers. The flower is very small, without petals. It has ovaries, pollen and female stigma separate lift shaft and stamens of 3, 6 or 8 (Figure 2.2).

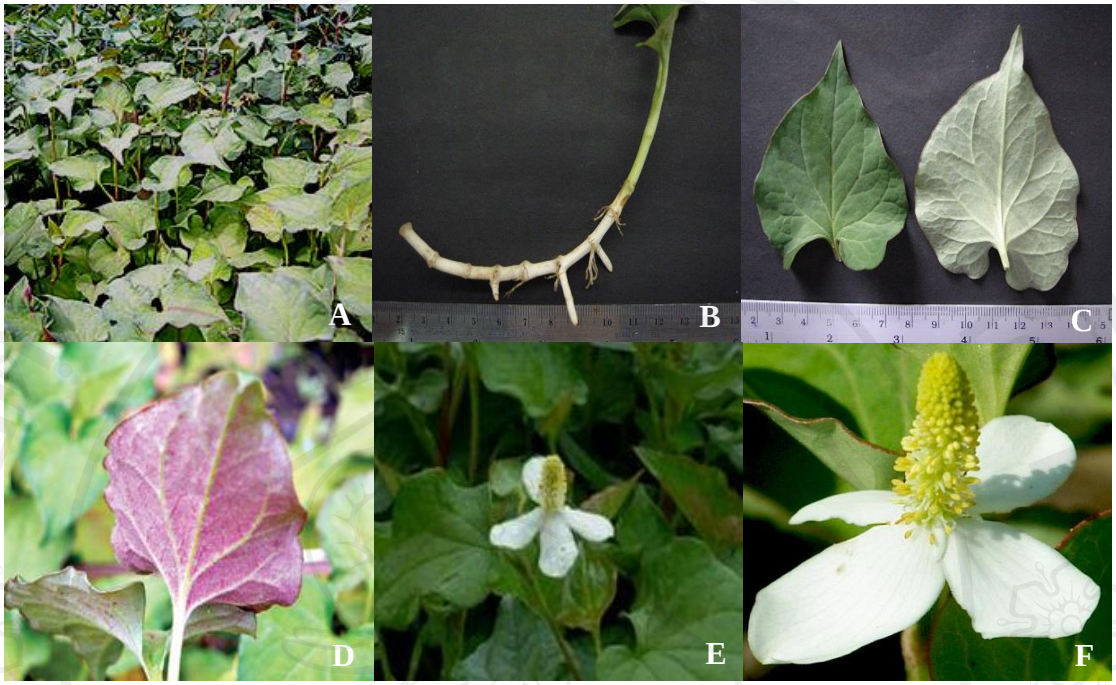

Figure 2.1 Botanical characteristics of Plookao. A) stem B) rhizomatous with a nodal segment C-D) leaves E-F) Flower

2.1.1 Taxonomy

For the taxonomic of plant, *Houttuynia cordata* Thunb. plant is classified into Kingdom Plantae, Subkingdom Racheobionta or Vascular plants, Superdivision Spermatophyta or Seed plants, Division Magnoliophyta**,** Class Magnoliopsida, Subclass Magnoliidae, Order Piperales, Family Saururaceae or Lizard's tail family, Genus *Houttuynia* Thunb. and Species *Houttuynia cordata* Thunb. The synonym name is *Polypara cochinchinensis* Lour. and *P. cordata* O.K. (Medicinal Plant Research Institute, 2003).

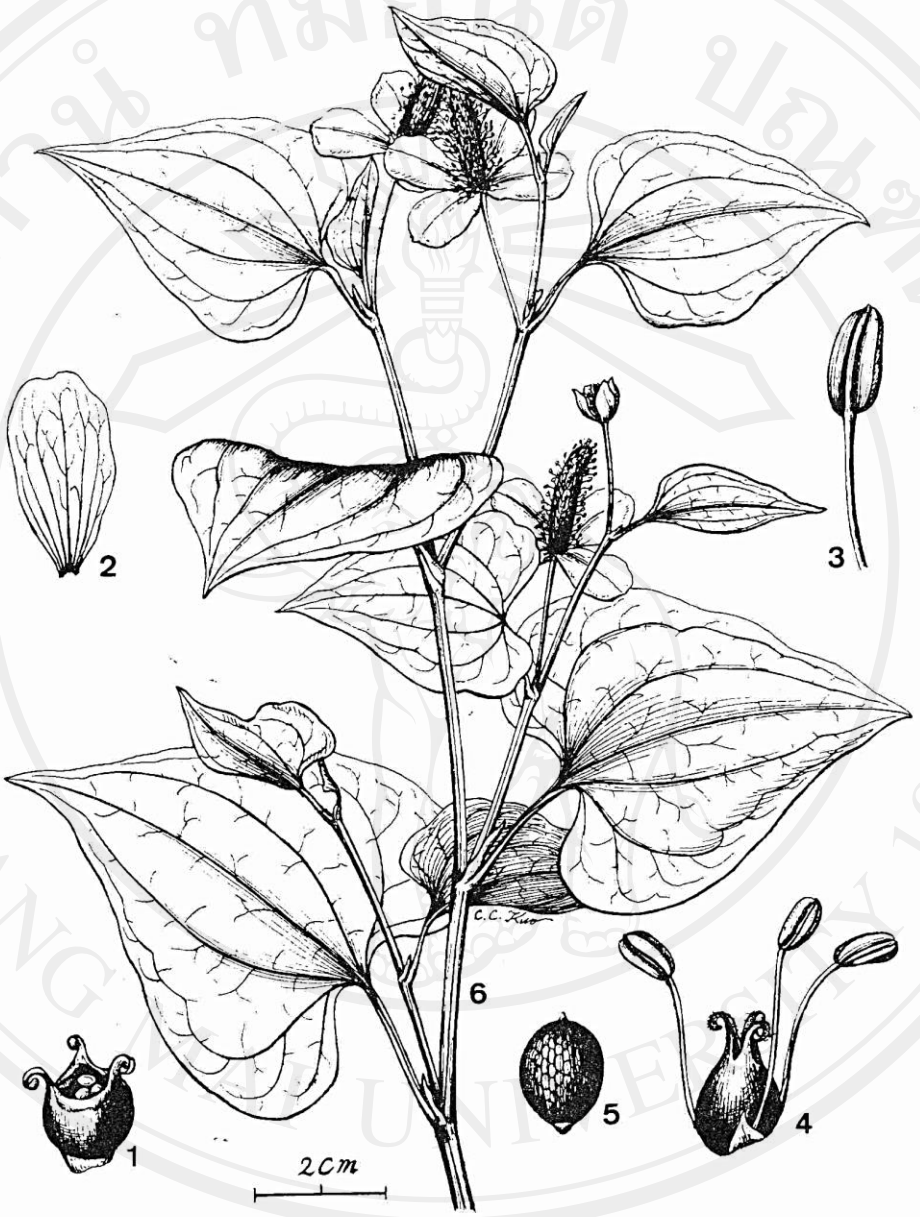

Figure 2.2 Details of keys of *Houttuynia cordata* Thunb. 1) dehicing 2) bract 3) stamen 4) flower 5) seed 6) habit
Source : Herbarium of National Taiwan University Digital Archives (No date)

2.1.2 Distribution

The *Houttuynia cordata* Thunb. plant grows well in moist to wet soil and even slightly submerged in water in partial or full sun (Wikipedia, 2009). Plookao vegetables have a general distribution in East and Southeast Asia. Nepal across to China and Japan and down through Southeast Asia up from the low plains to a height of about 2,500 meters above sea level. It is sometimes considered an invasive weed. The plant is more common in the north. It is also found as a herb in Central and North-East parts of Thailand. In the northern area of Thailand, Plookao is found in moist and shady places like ravines, riverbanks, forests, meadows, slopes, thickets and field margins and roadsides. Plookao plants need shade and high humidity. The plant can grow in different soils from the fertile loam soil, sandy soil with low nutrient content, and some grow in water. It is often grow near water sources, high humidity and an area that gets very little sunlight but it has the light throughout of the day. It is one of the vegetable eaten with traditional food in this area. Now a day, there are both natural and plantation farming available.

2.1.3 Utilization of Plookao

Plookao has been used as vegetable, food, cosmetic, herbal and age-old therapeutic among different communities of the region of Asia for long time. Leaves can be eaten raw or cooked. The weak point of this plant is its aromatic properties of rotten fish. As Plookao has been reported for high antioxidation property, novel application also concentrated on medicinal uses of this herbaceous species.

There have been many reports on medicinal applications of Plookao. The whole plant has antibacterial, antiphlogistic, depurative, diuretic, emmenagogue, febrifuge, laxative and ophthalmic. The leaves and stems are harvested during the growing season and used fresh in decoctions. A decoction is used internally in the treatment of many ailments including cancer, coughs, dysentery, enteritis and fever. It was a report on the induction of immune system by Plookao. Externally, it is used in the treatment of snakebites and skin disorders. The leaf juice is antidote and astringent. The root is also said to be used in medicinal preparations for certain diseases of women. A root extract is diuretic. The rhizomes yield a sterol, resembling sitosterol, which stimulates the secretion of antibiotic substances from a gram-positive

spore-forming bacillus. An active substance, effective in the treatment of stomach ulcers, has also been extracted from the plant (Global Invasive Species Database, 2009). For traditional folk, fresh and dried leaves, and roots of Plookao are used to dry the solution boils, swelling and inflammation of lung edema and inflammation. The twist is used in the urinary tract infections, middle ear infections and hemorrhoids. Fresh leaves are used as a poultice for external wounds, skin diseases, inflammation, swelling, sores and bites of poisonous snakes.

There are many reported on application of the plant recently. For example, the work of Weifeng *et al.* (2011) examined the effects of *Houttuynia cordata* on lipopolysaccharide (LPS)-induced prostaglandin (PG) E2 production, an indirect indicator of cyclooxygenase-2 (COX-2) activity. They found that COX-2 gene and protein expression in mouse peritoneal macrophages. The results showed that *Houttuynia cordata* was able to inhibit the release of LPS-induced PGE2 from mouse peritoneal macrophages (IC50 value: 44.8 µg/mL). Moreover, the inhibitory activity of *Houttuynia cordata* essential oil elicited a dose-dependent inhibition of COX-2 enzyme activity (IC50 value: 30.9 µg/mL). *Houttuynia cordata* was also found to cause reduction in LPS-induced COX-2 mRNA and protein expression, but did not affect COX-1 expression. The non-steroidal anti-inflammatory drug (NSAID) and specific COX-2 inhibitor NS398 functioned similarly in LPS-induced mouse peritoneal macrophages.

Lu *et al*. (2006) studied *Houttuynia cordata* injection (HCI), a traditional Chinese medicine used in China, it was chosen as one of eight types of traditional Chinese medicine that play a unique role in severe acute respiratory syndrome (SARS). The chemical composition of HCI has been analysed by GC-MS and the inflammation induced by carrageenan in the rat pleurisy model. The results showed that these parameters were attenuated by HCI at any dose and touched bottom at dose of 0.54 ml/100 g, although less strong than dexamethasone. This drug was also effective in inhibiting xylene induced ear edema, and the percentage of inhibition came to 50% at dose of 80 µl/20 g. The results clearly indicate that HCI have anti-inflammatory activity.

Eun *et al*. (2009) investigated the effects of *Houttuynia cordata* water extract (HCWE) on passive cutaneous anaphylaxis (PCA) in mice and on IgE-

mediated allergic response in rat mast RBL-2H3 cells. Oral administration of HCWE inhibited IgE-mediated systemic PCA in mice. The *in vivo/in vitro* anti-allergic effect of HCWE suggests possible therapeutic applications of this agent in inflammatory allergic diseases through inhibition of cytokines and multiple events of FceRI-dependent signaling cascades in mast cells.

The work of Lingmin *et al*. (2011) compared the antioxidant potential of a water-soluble polysaccharide (HCP) with solvent extracts from *Houttuynia cordata* Thunb. The results showed that polar water extract exhibited the highest reducing power and scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, superoxide radical and hydroxyl radical, which were correlated with its high level of biopolymer HCP. This finding suggests that HCP is one of the main active ingredients responsible for antioxidant effect of *Houttuynia cordata*, which might be valuable as a natural antioxidant source applied in both healthy medicine and food industry.

2.1.4 Secondary metabolite of Plookao

Plookao contains 6 major groups of important active ingredient that are volatile oil such as n-decylaldehyde and methyl n-nonyl ketone. This is used for inhibitory growth of virus cell culture such as HSV-1 and *influenza* virus. Then, flavonoid such as quercitin was used for vasoexpansion. A large group of alkaloids has been used for cytotoxicity against tumor cell lines, antileukemic activity, and for anti-imflamation. Plookao contains fatty acids and sterols (Medicinal Plant Research Institute, 2003). Finally, there is a group of other types such as Mineral fluoride. The chemical substance found from Plookao and their effect is shown in the following table.

Table 2.1 The chemical composition of 6 important different kinds of Plookao.

Actives ingredient	**Chemical substance**	**Effect**
1. Volatile oil	d-bormeol, bornyl Acetate camphene, Capryl aldehyde, α-caryophyllene, Caryophyllene oxid1, *p*-cymene decan-1-al, Decanoate methyl, 3,5-didecanoyl-4-nonyl, 3-deanoyl-4-nonyl-5-dodecanoyl, n-decyladehyde, 3,5-didodecaoyl-4-nonyl, Dodecan-1-al, dodecanaldehyde, Dodecanoate, Geraniol, Hexadecanoate, Methyl-n-nonyl ketone, Limonene, 2-nethythp-tenone, Methyl-n-noyl ketone, Myrcene, Octan-1-al, α-pinene, *b*-pinene, Thymol, Undeca-2-one, Vomifoliol	- Destroy cancer cell in the experiment tube - Stop the influenza virus raise cell - Stop HSV-1, influenza and HIV-1 in experiment tube - Anti staphylococcus and fungus - Stop growing of *H. influenza, pneumococcus, A. aureus*
2. Flavonoids	Afzelin, Hyperin, Isoquercetin Isoquercitrin, Quercetin, Quercitrin, Reynoutrin, Rutin , Afzelin, Hyperin, Isoquercetin , Isoquercitrin, Quercetin, Quercitrin, Reynoutrin, Rutin	To enlarge the vain

Table 2.1 The chemical composition of 6 important different kinds of Plookao. (Cont.)

Actives ingredient	**Chemical substance**	**Effect**
3. Alkaloids	Aristolactam A, Aristolactam A II, Aristolactam B, Ceparanone B, Cepharadione B, Cordarine, Norcepharadione B, Piperolactam A, 1,4-dihydropyridine, 2-nonyl-5-Decanoylpyridine, 3,5-didecanoylpyridine, Benzamide, Cis-N-(4-hydroxy-styryl), Benzamide trans-N-(4-Hydroxy-styryl)	- Destroy cancer cell in the experiment tube - Anti infection by stopping cyclooxygenase
4. Fatty acids	Capric acid, Lauric acid, Linoleic acid, Linolenic acid, Palmitic acid, Steric acid, Tetradecanoic acid	- Anti-inflammation from arthititis, diabetes and cancer - Protect cardiovascular disease
5. Sterols	Phytol, *b*-sitosterol, Spinasterol, Stigmasterol	- Effect on cardiovascular disease (CVD) or overall mortality exists
6. The other types	Chlorogenic acid, Mineral are fluoride, Potassium chloride, Potassium sulfate	- Anti-diabetic - Inhibits the release of glucose into the blood stream

Source : Herbal wine (2006), Joe *et al*. (2012), Biopanax (2013)

From a study by Minmin *et al*. (2005), they compared various sampling techniques for the gas chromatography–mass spectrometry of volatile constituents present in *Houttuynia cordata* Thunb. The result showed that (HCT) 2-Undecanone (22.21%) and houttuynum (7.23%) were predominant components of HCT samples obtained by HS-SPME whereas those levels were 3.95 and 3.60% in the same samples by FE and 25.93 and 6.60% in those by SD, respectively. Some minor constituents were isolated by SPME, but not by SD and FE. This carries great significance because of the importance of the oil volatiles to clinical therapy. They concluded that HS-SPME is a powerful tool for determining the volatile constitutes present in the TCMs.

2.2 Plant Tissue Culture

Plant *in vitro* culture techniques are essential to many types of academic inquiry, as well as to many applied aspects of plant science. In the past, plant tissue culture techniques have been used in academic investigation of totipotency and the roles of hormone in cytodifferentiation and organogenesis. Currently, tissue cultured plants that have been genetically engineered provide insight into plant molecular biology and gene regulation. Plant tissue culture techniques are used in applied plant science, including plant biotechnology and agriculture. In addition, the management of genetically engineered cells to from transgenic whole plant requires tissue culture procedures. For example, select plants can be cloned and culture as suspended cells from which plant products can be harvested. Tissue culture methods are required in the formation of somatic haploid embryos from which homozygous plans can be regenerated. Thus, tissue culture techniques have been, and still are, prominent in academic and applied plant science (Mineo, 1990)

Table 2.2 Some reports of successful plant tissue culture.

Plants	Summary	Reference
Acacia catechu Willd.	Callus was derived from cultured cotyledons on MS+(0.25)2,4-D+0.25NAA. Plantlets regenerated from the callus and nodal explants on MS+2.0BAP+2.0Kinetin. For rhizogenesis, microshoots of in length were dipped in sterilized 10IAA follow by tranfering to half strength MS+0.02% activated charcoal.	Thakur (2002)
Mucuna macrocapa Wall.	A highest growth of creamy compact cullus and maximum number of shoot multiplication development on MS media is better than WPM (wood plant media).	Wongsriwiwat (2007)
Caladium bicolor (Ait.) Vent. and *C. humboldtii* Schott.	Modified MS that have macronutrient from MS and micronutrient from RN (Ringe and Nitsch 1968) was used in micropropagation.	Somkanea (2007)
Lilium davidii var. unicolor	Haft Strength MS with PGRs was use for inoculate explant but the small shoot were inoculated on MS and the plant regeneration were conducted in NN basal medium.	Lingfei *et al*. (2008)
Rubus hybrid cultivar Black Satin	Three-month explants cultured on 0.02 mg / l TDZ produced a high regeneration frequency. The shoot primordia developed within 3 weeks from the point of the petiole from the leaf blade.	Sandhya and Mahalaxmi (2008)

Table 2.2 Some reports of successful plant tissue culture. (Cont.)

Plants	Summary	Reference
Kosteletzkya virginica	There was 93.94% callus induction on MS medium supplemented with 1.0 mg/l IAA, 0.3 mg/l kinetin. And 65.83% shoot induction on ½MS medium supplemented with 0.1 mg/1 IAA, 0.5 mg/1 zeatin. 96.67% rooting on MS medium.	Cheng-Jiang *et al*. (2008)
Zamioculcas zamiifolia Engl. (ZZ)	Leaf from the basal with or without petiolule or mid-rib that were placed vertically into the medium except for the explants with mid-rib from the basal part of the leaflet that were placed horizontally as well.	Papafotiouand Aekaterini N. Martini (2008)
Mucuna macrocapa Wall.	A highest growth of creamy compact cullus and maximum number of shoot multiplication development on MS media is better than WPM (wood plant media).	Wongsriwiwat (2007)

2.2.1 The advantages of plant *in vitro* culture

The advantages of plant *in vitro* culture is propagation large number of clone in short time and produce disease free plant**.** It is a basic method for plant improvement and plant metabolite production. Plant tissue culture is also used for biochemical, physiological and genetic study. Finally, the technique is necessary for germplasm storage of plants.

2.2.2 Factors effecting plant tissue culture

To achieve successful *in vitro* propagation, several factors has effect on plant tissue culture have to be optimized. These factors including

A. Culture Media and Plant Growth Regulators (PGRs)

- Culture media

Although more than 50 different devised media formulations have been used for *in vitro* culture of plant tissues for various plant species (Gamborg *et al*., 1976; Huang and Murashige, 1997). Basically, a nutrient medium consists of all the essential major and minor plant nutrient elements, vitamins, plant growth regulators and carbohydrate as carbon source with other organic substances as optional additives. A medium is identified by its mineral salt composition. Vitamins, hormone and other organic supplement vary widely with respect to composition and concentration. The choice depends on the plant species and to a degree upon the intended use of the culture (Trevor, 1981). Culture *in vitro* has to be transferred to new medium repeatedly to replenish nutrients or other substances in the medium.

Concentration of salts in the media composition may play an important role for plant regeneration. A wide variety of salt mixture has been reported in plant tissue culture medium. Several have been tested with callus and cell cultures of a wide variety of plant species.

One of the most commonly used media for plant tissue cultures is that developed by Murashige and Skoog (MS) medium (Texas Tech University, 2008). It is very high concentration of nitrate, potassium and ammonia. Many other media have been developed and modified. However, it is not always necessary to test many kinds of basal media when a callus is induced. It would be better to use only one or two kinds of basal media in combination of different kinds and concentrations of phytohormones. The most suitable medium composition should be optimized afterwards in order to obtain higher level of products as well as higher growth rate. As in cherry, half concentration MS macronutrients resulted in more growth than full or double concentration MS macronutrients resulted in more growth than full or double concentration (Ružić *et al*., 2003). The application of tissue transfer experiments between various culture media revealed a number of organogenesis was described with different types of culture media for each stage.

- Plant Growth Regulators (PGRs)

Plant hormones and growth regulators are chemicals that affect flowering, aging, root growth, distortion and killing of leaves, stems, and other parts, prevention or promotion of stem elongation, color enhancement of fruit, prevention of leafing and/or leaf fall; and many other conditions. Very small concentrations of these substances produce major growth changes.

In general, hormones are produced naturally by plants, while plant growth regulators are applied to plants by humans. Plant growth regulators may be synthetic compounds, for example IBA and Cycocel, that mimic naturally occurring plant hormones, or they may be natural hormones that were extracted from plant tissue such as IAA.

Applied concentrations of these substances usually are measured in parts per million (ppm) and in some cases parts per billion (ppb). These growth-regulating substances most often are applied as a spray to foliage or as a liquid drench to soil around a plant's base. Generally, their effects are short lived, and they may need to be reapplied in order to achieve the desired effect.

There are five groups of plant-growth-regulating compounds that are auxin, gibberellin (GA), cytokinin, ethylene, and abscisic acid (ABA). For the most part, each group contains both naturally occurring hormones and synthetic substances.

The importance of plant growth regulators in plant tissue culture is well documented. Phytotechnology offers a broad range of plant growth regulators specifically tested for plant cell culture. Each commercial product is assayed for physical and chemical characteristics then is biologically tested following the criteria established for powdered media. Basically, auxin is tested for enhancement of callus growth and/or root initiation *in vitro* whereas cytokinin is tested for stimulation of shoot production.

Auxins are generally used in plant cell culture at a concentration range of 0.01-10.0 mg/L. When added in appropriate concentrations they may regulate cell elongation, tissue swelling, cell division, formation of adventitious roots, inhibition of adventitious and axillary shoot formation, callus initiation and growth, and induction of embryogenesis.

Cytokinins are generally used in plant cell culture at a concentration range of 0.1-10.0 mg/L. When added in appropriate concentrations they may regulate cell division, stimulate auxiliary and adventitious shoot proliferation, regulate differentiation, inhibit root formation, activate RNA synthesis, and stimulate protein and enzyme activity.

Gibberellins are generally used to promote stem elongation, flowering, and breaking dormancy of seeds, buds, corms, and bulbs. There are over 90 forms of gibberellins, but GA3 is the most commonly used form. Compounds like paclobutrazol and ancymidol inhibit the synthesis of gibberellins.

Abscisic Acid (ABA) plays a role in dormancy development in embryos, buds and bulbs, and in leaf abscission. When used in tissue culture, ABA inhibits the growth of shoots and the germination of embryos. Fluridone may inhibit ABA synthesis. Polyamines: Polyamines are compounds that occur in high levels within plants and are used in tissue culture media at concentrations of 10-1000 mM. Polyamines may enhance regeneration of roots, shoots and embryos, delay or prevent senescence, and regulate flowering.

Table 2.3 Show the function of plant growth regulators in plant tissue culture

PGR Class	Product Name	Function in Plant Tissue Culture
Auxins	Indole-3-Acetic Acid Indole-3-Butyric Acid Indole-3-Butyric Acid, K-Salt α-Naphthaleneacetic Acid α-Naphthaleneacetic Acid, K-Salt 2,4-D (Solutions) ρ-Chlorophenoxyacetic acid Picloram Dicamba	Adventitious root formation (high concentration) Adventitious shoot formation (low concentration) Induction of somatic embryos Cell Division Callus formation and growth Inhibition of axillary buds Inhibition of root elongation
Cytokinins	6-Benzylaminopurine6-(γ,γ-Dimethylallylamino)purine (2iP) 2iP-2HCl Kinetin Thidiazuron (TDZ) N-(2-Chloro-4-pyridyl)-N-phenylurea Zeatin Zeatin Riboside	Adventitious shoot formation Inhibition of root formation Promotes cell division Modulates callus initiation and growth Stimulation of axillary bud breaking and growth Inhibition of shoot elongation Inhibition of leaf senescence
Gibberellins	Gibberellic Acid (GA3) GA4/7	Stimulates shoot elongation Release seeds, embryos, and apical buds from dormancy Inhibits adventitious root formation
Abscisic Acid	Abscisic Acid	Stimulates bulb and tuber formation Stimulates the maturation of embryos Promotes the start of dormancy, leaf abscision

Table 2.3 Show the function of plant growth regulators in plant tissue culture (Cont.)

PGR Class	Product Name	Function in Plant Tissue Culture
Polyamines	Putrescine Spermidine	Promotes adventitious root formation Promotes somatic embryogenesis Promotes shoot formation
Antimitotics	Colchicine Oryzalin Trifluralin	Binds to the tubulin dimers during cell division thus preventing the formation of spindle fibers; this results in doubled chromosomes
Dwarfing Agents/ "Anti-GA's"	Ancymidol CCC Paclobutrazol Trinexapac-Ethyl	Interferes with gibberellin synthesis or activity Reduces internodal elongation Promotes tuber,corm and bulb formation

There are several reports on plant tissue culture using combination of plant growth regulators. Almasri, 2000 had indicated that *Podocarpus gracilior* was successfully developed a large number of shoots when cultured on modified WPM supplemented with BAP 0.5 mg/l. The most roots were developed on modified WPM supplemented with IBA 0.5 mg/l.

In an article by Mehta (2000) mature zygotic embryo axis of tamarind (*Tamarin dusindica*) consists of longituditional section attached to cotyledons were cultured in a modified MS medium supplemented with NAA 0.5 mg/l, BAP 10.0 mg/l and sucrose 4% (w/v) to induce adventitious shoot buds.

Kulkarni and D'Souza (2000) reported that *in vitro* shoot tip of *Butea monosperma*, Four to five multiple buds were induced from the axis of the cotyledonary nodes, with cotyledons intact, on 1/2 strength WPM supplemented with BAP 5.0 mg/l alone and sucrose 30 g/l.

A study by Kulneet and Kant (2000) showed that *in vitro* micropropagation through shoot apices of *Acacia catechu*, explants were excised from 15-day-old in vitro grown seedlings raised from superior seed stocks. Shoot bud

induction from shoot apex explants had a maximum of 12 shoots was obtained on MS medium supplemented with BAP 1.5 mg/l and Kinetin 1.5 mg/l. Well-developed shoots (3–4 cm long) were rooted on 1/4 strength MS medium with IAA 3.0 mg/l and sucrose 1.5%.

The work of Sasmitamihardja *et al.* (2001) revealed that *in vitro* regeneration of *Paraserianthes falcataria* (L.) Nielsen was achieved through axillary shoot multiplication. Single-node segments from six-day-old seedlings were used as explants and cultured on modified MS medium. The addition of IAA 0.01 - 1.0 μM in combination with BA 0.1 - 1.0 μM induced axillary shoot proliferation. During the first two weeks in culture, 4-15 shoots per explants were proliferated. The highest number of shoots was obtained from single-node explants cultured on the medium supplemented with IAA 0.1 μM and BA 0.5 μM. After three weeks in culture, up to 23 shoots per explants were produced. Lengthening the period of culture produced no significant increase in shoot proliferation. In the last week of a 4-week period of culture, the shoot multiplication rate was very low. Regenerated shoots were elongated on MS medium without growth regulators.

Vengadesan *et al* (2000) had drawn attention to the fact that many species in the genus Acacia, one of the important genera of the family Fabaceae, are more commonly micropropagated by using field explants. Cotyledonary nodes, epicotyl and shoot tip explants were used to induce multiple shoots; however, their data revealed that nodal explants were most suitable for multiple shoot formation. The WPM used commonly for the *in vitro* propagation of woody species had a very little impact on Acacia species. MS medium was preferred in many of the Acacia species for shoot induction, elongation and root induction. In some cases activated charcoal at 2.0 mg/l was used as an additive to prevent callusing, leaf chlorosis and to improve elongation and rooting. In the genus Acacia, frequent subculture of explants at constant intervals (25-30 days) made significant improvement in enhancing the number of multiple shoots.

Liu *et al*. (2002) reported that *P. lobata* hairy roots have faster elongation and more branches than normal roots. The responses of hairy roots and normal roots to treatment with IAA, IBA, and NAA were different. In normal roots, all the three auxins strongly stimulated lateral root formation at all tested concentrations.

Responses to IAA and IBA in primary root growth and lateral root elongation were similar and depended on concentration; promotion at 0.1 μM, no effect at 1.0 μM, and inhibition at 2.5 μM.

Purohit *et al.* (2002) pointed out that multiple shoots of *Quercus leucotrichophora* L. and *Q. glauca* Thunb. were induced from the intact embryos (decoated-seeds) as well as from the cotyledonary nodes (with attached cotyledons but without radicle and primary shoot) of 3 weeks old *in vitro* grown seedlings on WPM and MS media supplemented with BA, either alone or in combination with GA3, IBA. BA at 5.0 mg/l was effective for induction of multiple shoots, and addition of GA3 to the medium further enhanced the shoot number and shoot height but resulted in shoot thinness. High frequency shoot multiplication was achieved using cotyledonary nodes. Shoots were further multiplied from the original explants on WPM supplemented with BA 5.0 mg/l. Nearly 78% and 67% rooting were obtained in *Q. leucotrichophora* and *Q. glauca* microshoots (3–4 cm high), respectively on 1/2 strength WPM supplemented with 3.0 mg/l IBA. However, this was associated with basal callus formation. Treatment with IBA 5.0 - 20.0 mg/l for 24 or 48 h followed by transferring to plant growth regulator-free 1/2 strength WPM not only improved the rooting percentage but also avoided basal callus formation. IBA at 20.0 mg/l for 24 h was most effective (90% and 100% rooting in *Q. leucotrichophora* and *Q. glauca*, respectively). *In vitro* rooted plants were hardened and established in garden soil.

Al-Wasel (2002) reported that *in vitro* mass propagation of *Acacia seyal* using seedling shoot tip explants was achieved on MS medium supplemented with BA in the presence of NAA. The greatest shoot multiplication with long shoots was observed on the media containing BA 2.0 mg/l with NAA 0.1 or 0.5 mg/l and BA 4.0 mg/l with NAA 0.1 mg/l; with mean number of shoots at 6.4 and 6.7, respectively. Microshoots were rooted better on 1/2 MS medium supplemented with IBA. The highest rooting percentage (80%) and root number (4.9 roots / microshoot) were promoted by IBA 4.0 mg/l. The plantlets (90%) successfully survived acclimatization *ex vitro*.

The work of Thakur *et al.* (2002) asserted that a callus was derived from cultured cotyledons on MS medium supplemented with 2,4-D 0.25 mg/l and NAA 0.25 mg/l. Plantlets regenerated from the callus and nodal explants on MS medium

containing BAP 2.0 mg/l and Kinetin 2.0 mg/l were further multiplied on the same medium. Addition of adenine sulphate 25.0 mg/l, ascorbic acid 20.0 mg/l and glutamine 150.0 mg/l in the medium resulted in enhanced axillary branching. Multiple shoots formed after 6 weeks were separated and subcultured in the fresh medium of same composition. For rhizogenesis, microshoots of 2.0-2.5 cm in length were dipped in sterilized IAA 10 mg/l solution for 24 h followed by transferring to half strength MS medium containing activated charcoal (0.02%), resulting in rooting (75%) within 8 weeks. The rooted plants were transferred to pots containing sterilized soil and sand mixture for hardening and 71% survival was recorded. Fifty true-to-type plantlets of *A. catechu* could be obtained within seven months of culture establishment.

A study by Nanda (2003) showed that from *in vitro* propagation of *Acacia mangium*, a tropical leguminous tree, bud sprout was obtained from mature nodal explants of 10-year-old tree on MS basal medium supplemented with BAP 1.0 mg/l, GA31.0 mg/l and IAA 0.05 mg/l. The rate of multiplication was obtained on MS medium supplemented with BAP 1.5 mg/l, IAA 0.05 mg/l and adenine sulfate 100 mg/l. The multiplication rate varied from 1 to 8, depending on the plant growth regulators used. Excised shoots were rooted on 1/2 strength MS basal salts supplemented with IBA 0.5 mg/l or IAA and sucrose 20 g/l (w/v) after 13-14 days of culture.

Thiem (2003) indicated that *in vitro* clonal propagation of a valuable medicinal plant, i.e. *P. lobata* by enhancing axillary bud proliferation in shoot tip explants achieved on MS medium supplemented with Kinetin 1.0 mg/l and IAA 1.0 mg/l, yielding an optimum frequency of shoot formation (94%) and shoot number (3.6 shoots per explants). The best shoot elongation was obtained on the MS medium containing GA3 2.0 mg/l and BA 1.0 mg/l. Rooting was the highest (100%) on the full-strength MS medium with NAA 0.5 mg/l, IAA 0.5 or 2.0 mg/l.

According to Minh (2005) *Aquilaria crassa* (agarwood), a Vietnamese forest tree, was micropropagated using shoot explants from 20-year-old trees known to produce the valuable exudates ‘tok’. Either shoot tips or internodes could be used for the initial explants, but in subcultures best results were obtained from internodes. WPM was a better basal medium than MS, and for initial shoot induction BA 1.0 mg/l and coconut water at 10% (v/v) were used. For subcultures, BA 0.1 mg/l, NAA 0.1

mg/l and coconut water at 10% gave highest shoot multiplication. A low level of rooting was obtained using either IBA or NAA at 0.3 mg/l. Plants transferred to the field grew to 2 m after 18 months and had normal morphology.

According to Lyyra *et al*. (2006) *in vitro* regeneration of black willow plants (*Salixnigra* Marsh.) could be obtained by using unexpanded inflorescence explants excised from dormant buds. The highest shoot regeneration frequency (36%) was achieved on BAP 0.5 mg/l. Mean number of shoots per explant varied from one to five. The ability of black willow inflorescences to produce adventitious shoots makes them potential targets for Agrobacteriummediated transformation with heavy-metal-resistant genes for phytoremediation.

Kartsonas and Papafotiou (2007) discovered that using nodal explants from seedlings in micropropagation of *Quercus euboica* gave higher multiplication rates than explants from adult plants. WPM salts, with myoinositol 100 mg/l, thiamine 1.0 mg/l, pyridoxine 0.5 mg/l, nicotinic acid 0.5 mg/l and sucrose 3% was used as basal medium and several cytokinins at various concentrations were evaluated for their effect on shoot multiplication. The highest shoot multiplication rate was obtained from BA 1.0 mg/l. But IBA 2.0 mg/l in the culture medium during the first week of culture, and followed by culturing in a plant growth regulator-free medium, gave the best rooting results. Darkness at the beginning of the rooting period did not improve rooting. The use of plastic wrap as a cover material for the culture vessels enhanced rooting percentage and root number. Plantlets acclimatized *ex vitro* in soil from the natural environment where the species grow survived at a higher percentage (up to 93%) and had more vigorous growth than those grown in a compost–perlite (2:1 v/v) medium (only 36%).

B. Explants

Various types of explants have been use in tissue culture including leaf, stem, shoot, root, seed, bult, bud and embryo. Very small piece of explants tissue, or even isolate individual cells, can be cultured and regenerated. The tissue has to be sterilized so it will not have any contaminating bacteria or fungus. It is then placed inside the tissue culture contain on media agar. Sugar, nutrients and hormones that the plant needs will be all dissolved in the agar for solid culture.

Kulkarni and D'Souza (2000) reported that *in vitro* shoot tip of *Butea monosperma*, Four to five multiple buds were induced from the axis of the cotyledonary nodes, with cotyledons intact, on 1/2 strength WPM supplemented with BAP 5.0 mg/l alone and sucrose 30 g/l. A study by Kulneet and Kant (2000) showed that *in vitro* micropropagation through shoot apices of Acacia catechu, explants were excised from 15-day-old *in vitro* grown seedlings raised from superior seed stocks. Shoot bud induction from shoot apex explants was observed on MS medium containing various growth regulators. A maximum of 12 shoots was obtained on MS medium supplemented with BAP 1.5 mg/l and Kinetin 1.5 mg/l. Well-developed shoots (3–4 cm long) were rooted on 1/4 strength MS medium with IAA 3.0 mg/l and sucrose 1.5%.

Al-Wasel (2002) reported that *in vitro* mass propagation of *Acacia seyal* using seedling shoot tip explants was achieved on MS medium supplemented with BA in the presence of NAA. The greatest shoot multiplication with long shoots was observed on the media containing BA 2.0 mg/l with NAA 0.1 or 0.5 mg/l and BA 4.0 mg/l with NAA 0.1 mg/l; with mean number of shoots at 6.4 and 6.7, respectively. Microshoots were rooted better on 1/2 MS medium supplemented with IBA. The highest rooting percentage (80%) and root number (4.9 roots / microshoot) were promoted by IBA 4.0 mg/l. The plantlets (90%) successfully survived acclimatization *ex vitro*.

Saswati *et al*. (2006) studied and reported an efficient and direct shoot bud differentiation and multiple shoot induction from nodal segments of underground stoloniferous rhizomes of *Houttuynia cordata* Thunb. The frequency of shoot bud regeneration was influenced by the type of cytokinin and concentrations. The number of shoots per explant was higher (20.00 ^ 2.61) on Murashige and Skoog (MS) medium supplemented with Kn (18.58mM) compared to BAP and 6-g,g-(dimethyl-allylamino)-purine (2iP) during initial 40-d-old culture. Subsequent shoot differentiation and multiplication were achieved in MS medium containing 9.29mM Kn and 15% (v/v) coconut milk. Elongation and growth of multiple shoots were also obtained on MS medium containing either 2.32 mM Kn or 2.46mM 2iP alone. The rate of shoot multiplication during subcultures declined with an increase in the size of proliferating shoot cluster.

C. Explants sterilization

Explants are surface-sterilized to avoid microorganism growth, which is detrimental to culture growth, before they are used to establish *in vitro* axenic cultures. There are 4 sources of infections that are the plant, including both internal and external sources, the nutrient medium which is insufficiently sterilized, the air, and the research workers that are inaccurate work, and the most important of these is the plant itself. Plant material should be well sterilized before being isolated *in vitro* (Pierik, 1997)

Many different materials have been used to surface disinfect explants, but the most commonly used are 1% (v/v) sodium hypochlorite (commercial bleach contains 5% sodium hypochloride), 70% alcohol, or 10% hydrogen peroxide. Other include using a 7% saturated solution of calcium hypochlorite, 1% solution of bromine water, 0.2% mercuric chloride solution, and 1% silver nitrate solution (Robert and Dennis, 2000)

2.2.3 Secondary production by plant tissue culture

Secondary metabolite in a plant plays a major role in the survival of the plant in its environment (Wink, 1988). The secondary metabolites formed are also an important trait (taste, color, scent) for food and ornamental plants. Moreover, numerous plant secondary metabolites such as alkaloids, anthocyanins, falvonoids, quinines, lignanes, steroids and terpenoids have found commercial application as drug, dry, flavor, fragrance and insecticides.

These fine chemicals are extracted and purified from plant materials and some are used in large amounts such as quinine and hop bitter acids with a world market of respectively about 500 and 7,500 tones annually. However, other such as vincriatine and paclitaxel has been traded just a few kilograms a year.

Natural resources of both potential and established secondary metabolite are vast. Some secondary metabolite compounds are extracted from easily available sources, such as agricultural and horticultural crops (maize, buckwheat, grapevine, carrots, beetroot, citrus hesperidia, hops, apples, berries, tea leaves) or medicinal plants such as pine, skullcap, sage, rosemary, and tormentil (González-Paramás *et al*.,

2004; Obied *et al*., 2005). However, production of plant secondary metabolite by classical practice is not always satisfactory. It is often restricted to a species or genus and might be activated only during a particular growth or developmental stage, or under specific seasonal, stress or nutrient availability conditions. For example some plants are difficult to cultivate, necessitating collection in the wild and thus the risk of the plant getting extinct. Some plants grow very slowly such as Cichona trees that need about 10 years before ready for harvesting thus requiring long term planning of possible market demands.

Phamaceutical products from plants is also need to be restrict to the GMP-rules. These reasons make a lot of effect to develop plant cell culture as a possible production method for plant secondary metabolites of commercial interest in the past year (Verpoorte *et al*., 1993; Su, 1995; Alfermann and Peterson, 1995)

These restrictions lead to a need for biotechnology to produce complex and expensive substances using tissue culture method. Conditions that make using biotechnological methods necessary for the production of secondary plant metabolites economically including high economic value, insufficient abundance in intact plants, limited availability from natural sources (for example; rare, endangered, and overexploited species), and finally difficult cultivation (Misawa, 1994; Verpoorte *et al.,* 2002). When taking these factors into consideration, *in vitro* technology offers some or all of the following benefits. It is simpler extraction and purification from interfering matrices. Novel products not found in nature, independence from climatic factors and seasons, more control over biosynthetic routes for obtaining the most desired variants (e.g. enantiomers or glycosides) or a proportion of given compounds, shorter and more flexible production cycles, easier fulfillment of the high-profile pharmaceutical production demands (such as GLP and GMP) can be produced by *in vitro* culture. Moreover, it is a method for exploitation of the genetic engineering potential, and for avoiding legal restrictions against GMOs introduction into the natural environment. As a demand for more specific secondary metabolite products that could be used in accurately defined therapeutic and nutritional situations is increasing, *in vitro* culture was also a way to investigate into the biosynthetic routes and advances our understanding of the function and regulation of plant metabolites (Grassman *et al.,* 2002; Misawa, 1994). It is reported that essential oils can be

prepared Plookao cream protects the skin rough. To meet the desired therapeutic effect it is indispensable to use the proper parts and procedures of the Plookao plant. The chemical composition is very different. In addition, some active ingredients are not stable when heated.

There has been able to generate industrially feasible in a curtain plant species. Pure compounds such as shikonin, taxol and berberine, or biomass such as ginseng roots have been reported based on *in vitro* method. However, for many of the pharmaceuticals of interest, the production is too low or even zero in the cultured cell, despite extensive studies on the optimization or growth and production media and cell line selection for high producing strain. This is usually due to the reason that production is controlled in tissue specific manner and dedifferentiation resulting thus inn loss of the production capacity. Therefore other approaches such as the various elicitor has been used (Verpoorte *et al*., 1991). In some cases, different cells produce the same products as the plant itself, but the large - scale production remains a bottleneck for the economy of such a production.