การเพาะเลี้ยงเนื้อเยื่อชะเอมเทศ (*Glycyrrhiza glabra* Linn.) และการตรวจสอบกลีเซอร์ไรซิน

นายวันฉัตร แสวงศักดิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2552

TISSUE CULTURE OF *Glycyrrhiza glabra* Linn. AND GLYCYRRHIZIN DETECTION

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	TISSUE CULTURE OF *Glycyrrhiza glabra* Linn. AND GLYCYRRHIZIN DETECTION
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วันฉัตร แสวงศักดิ์ : การเพาะเลี้ยงเนื้อเยื่อชะเอมเทศและการตรวจสอบกลีเซอร์ไรซิน (Tissue culture of *Glycyrrhiza glabra* Linn. and glycyrrhizin detection) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ดร.เพชรรัตน์ จันทรทิณ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร.อภิชาติ กาญจนทัต, 89 หน้า.

ชะเอมเทศ *Glycyrrhiza glabra* Linn. เป็นไม้ยืนต้นที่พบในทางตอนใต้ของยุโรปและบางส่วนของเอเชีย ซึ่งถูกนำมาใช้เป็นสารให้ความหวานแทนน้ำตาล และมีฤทธิ์ทางเภสัชวิทยา เช่น ต่อต้านการติดเชื้อและ ต้านไวรัส เป็นต้น การศึกษานี้เป็นการพัฒนาวิธีการขยายพันธุ์ชะเอมเทศเพื่อให้ได้ต้นจำนวนมากจากส่วนปลายยอดที่ได้จากการเพาะเลี้ยงในสภาพปลอดเชื้อ โดยศึกษาชนิดของสูตรอาหาร ความเข้มข้นของเกลือแร่และน้ำตาล ชนิดและความเข้มข้นของสารควบคุมการเจริญเติบโตกลุ่มออกซินและไซโตไคนิน ที่มีผลต่อการเจริญเติบโตและการพัฒนาของยอดในอาหารเพาะเลี้ยง จากการทดลองพบว่า สูตรอาหารที่เหมาะสมสำหรับการเจริญเติบโตและพัฒนาของต้นชะเอมเทศ คือ อาหารสูตร B5 ที่ความเข้มข้นของเกลือแร่ 1/2 เท่า และมีน้ำตาล 30 กรัมต่อลิตร อย่างไรก็ตามอาหารสูตร MS มีอัตราการเพิ่มปริมาณต้นได้ดี โดยอาหารสูตรนี้ที่เติม BA ที่ความเข้มข้น 0.5 มิลลิกรัมต่อลิตร สามารถเกิดยอดได้มากที่สุดคือ 4.75 ยอดต่อชิ้นส่วนพืช ส่วนสูตรอาหารที่สามารถชักนำให้เกิดรากอย่างมีประสิทธิภาพมากที่สุดคือ อาหารสูตร B5 ที่ความเข้มข้นของเกลือแร่ 1/2 เท่า ที่เติม IAA หรือ IBA ความเข้มข้น 5.0 มิลลิกรัมต่อลิตร หลังจากการเพาะเลี้ยงเป็นเวลา 6 สัปดาห์ จากการนำต้นที่มีรากสมบูรณ์ย้ายออกปลูกในสภาพธรรมชาติพบว่า มีการรอดชีวิต 95 เปอร์เซนต์ เมื่อใช้ดิน หรือเวอร์มิคูไลท์เป็นวัสดุปลูก สำหรับการวิเคราะห์ปริมาณสารกลีเซอร์ไรซินด้วยวิธี HPLC พบว่า การเลี้ยงในอาหารสูตร B5 ที่มีความเข้มข้นของเกลือแร่ในอาหาร 1/2 เท่า และเติม IAA ความเข้มข้น 5.0 มิลลิกรัมต่อลิตร ให้ปริมาณสารกลีเซอร์ไรซินมากที่สุดคือ 27.57±0.66 ไมโครกรัมต่อกรัมน้ำหนักแห้ง เปรียบเทียบกับกลุ่มควบคุมที่ไม่เติมสารควบคุมการเจริญเติบโต (13.66±1.21 ไมโครกรัมต่อกรัมน้ำหนักแห้ง)

สาขาวิชา: เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต วันฉัตร แสวงศักดิ์

ปีการศึกษา: 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก

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WANCHAT SAWAENGSAK : TISSUE CULTURE OF *Glycyrrhiza glabra* Linn. AND GLYCYRRHIZIN DETECTION. THESIS ADVISOR : PETCHARAT CHUNTARATIN, Ph.D., THESIS CO-ADVISOR: APHICHART KARNCHANATAT, Ph.D. 89 pp.

Licorice (*Glycyrrhiza glabra* Linn.) is a perennial plant native to Southern Europe and parts of Asia, used as a non-nutritional sweetener but with numerous reported pharmacological effects, including anti-inflammatory and antiviral properties. The study was aimed to optimize an *in vitro* micropropagation protocol of *G. glabra* plants from *in vitro* shoot tips, the effect of medium types, medium strength of salt base, sucrose concentration, cytokinin and auxin types were tested for the ability to support the growth of shoot tips in culture. The most suitable medium for *G. glabra* plant growth and development was 1/2 - strength B5 salt and 30 gL^{-1} sugar. However, MS medium supported a superior proliferation rate. MS medium supplemented with 0.5 mgL^{-1} of BA produced the maximum of shoots (4.75) per explant. The highest efficiency of root formation occurred in the 1/2-strength B5 medium containing 5.0 mgL^{-1} of either IAA or IBA after six weeks of culture. The survival rate of plantlets was 95% when used either garden soil or vermiculite as substrate culture. The glycyrrhizin contents were analyzed by HPLC. The production of glycyrrhizin in culture using 1/2- strength B5 medium supplemented with 5.0 mgL^{-1}IAA increased with time up to week 8 reaching 27.57±0.66 $\mu g\ g^{-1}$ dry wt as compared with control (13.66±1.21 $\mu g\ g^{-1}$ dry wt).

Field of study: Biotechnology Student's signature: Wanchat Sawaengsak
Academic year: 2009 Advisor's signature: Petcharat Chuntaratin
Co-advisor's signature: Aphichart Karnchanatat

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MS	=	Murashige and Skoog medium (1962)
B5	=	Gamborg medium (1968)
WPM	=	Llyod and McCown (1981)
BA	=	6-Benzyladenine
NAA	=	α-naphthaleneacetic acid
Kn	=	Kinetin
IAA	=	Indole-3-acetic acid
IBA	=	Indole-3-butyric acid
TDZ	=	Thidiazuron
cm	=	centrimeter(s)
µg	=	microgram(s)
g	=	gram(s)
mgL^{-1}	=	milligram per liter
ml	=	milliter
hrs	=	hour(s)
temp	=	temperature (°C)
v/v	=	volume/volume (concentration)
w/v	=	weight/volume (concentration)
µl	=	microliter(s)
HPLC	=	High performance liquid chromatography
GA	=	Glycyrrhizic acid

CHAPTER I

INTRODUCTION

In Thailand today, *Glycyrrhiza glabra* Linn., commonly called licorice has been imported from China. As a result of restriction of specific ecological requirement that is not naturally cultivated. However, the increased use of this plant in recent years has yet to be realized, this trend is expected to increase as field cultivation in Thailand surge on. Licorice has been used as a sweetener and flavoring agent in candies industry as well as an important drug in oriental medicine. Glycyrrhizin is obtained from the roots and tubers of field grown intact plants, but the ability to grow the plant is restricted to certain climates only. In addition, the glycyrrhizin content may well vary with environmental changes, between seasons, soils and agricultural conditions, as well as between cultivar genetics. Even within the problem of these variations lines the more serious logistics of culture since the conventional method for propagation of *G. glabra*, a cross-pollinated plant, is of course via seed, yet the plant typically has poor seed viability (germination potential) which restricts its multiplication and thus ease of economically viable agriculture. To increase substantial quantities and derive a maximum benefits from this plant, it is necessary to propagate the plantlets. *In vitro* micropropagation by mean of tissue culture techniques is a powerful tool for rapid clonal multiplication of species in which conventional methods have limitations. In addition to plant tissue culture is an attractive alternative source to whole plant for the production of high value secondary metabolites.

Although, several reports on tissue culture of licorice have been published but lacked the detail of culture media studies and analytical procedures. To start to clarify this situation, this research reports on a study which is aimed to evaluate the optimal culture media and plant growth regulators on shoot multiplication and root induction for glycyrrhizin production by tissue culture techniques.

CHAPTER II

LITERATURE REVIEWS

2.1 Historical Reviews of *Glycyrrhiza glabra* Linn.

Glycyrrhiza glabra Linn., commonly known as 'licorice', belongs to the family Leguminosae in the genus Glycyrrhiza which contains 30 species native to subtropical and hot temperate regions. The licorice plant, as in *G. glabra*, is a herbaceous perennial native to Southern Europe and parts of Asia, the roots and rhizomes of which have been used commercially as non-nutritional sweetening and flavoring agent in some candies and pharmaceuticals formulations. The principal active component in licorice extract is Glycyrrhizin which is localized exclusively in the underground woody parts of the thickening roots and stolons (rhizomes) where it can comprise from 2-14% of the dry weight whereas soya-saponins, which are also oleanane-type triterpene saponins, are localized mainly in the seeds and rootlets of the licorice plant (Hayashi *et al*., 1996). The chemical structure of glycyrrhizin is shown in Figure 2.1. The roots of the licorice plant (*G. glabra*) are one of the oldest known botanicals species in Chinese medicine where the beneficial health properties attributed to licorice include anti-inflammation, laxative, immunomodulatory, anti-ulcer, anti-allergy and anti-carcinogenesis (He *et al*., 2001; Matsui *et al*., 2004; Takahara *et al*., 1994; Wang and Nixon, 2001). In addition, glycyrrhizin has anti-cancer, anti-bacterial, anti-spasmolytic and anti-viral activities, the latter against both DNA and RNA viruses (Fiore *et al*., 2008). Recently, it has been found to be highly active in inhibiting replication of the Severe Acute Respiratory Syndrome (SARS)-associated virus as well as a potential therapeutic agent for chronic hepatitis and Acquired Immuno Deficiency Syndrome (AIDS) (Baba *et al*., 1988; Cinatl *et al*., 2003; Ito *et al*., 1987).

Glycyrhizin is obtained from the roots and tubers of field grown intact plants, but the ability to grow the plant is restricted to certain climates only. In addition, the glycyrrhizin content may well vary with environmental changes, between seasons, soils and agricultural conditions, as well as between cultivar genetics. Even within the problem of these variations lines the more serious logistics of culture since the

conventional method for propagation of *G. glabra*, a cross-pollinated plant, is of course via seed, yet the plant typically has poor seed viability (germination potential) which restricts its multiplication and thus ease of economically viable agriculture. The alternative source which offers the opportunity to overcome the poor seed germination problem is *in vitro* culture which can provide a rapid propagation of new varieties within an economically viable timescale.

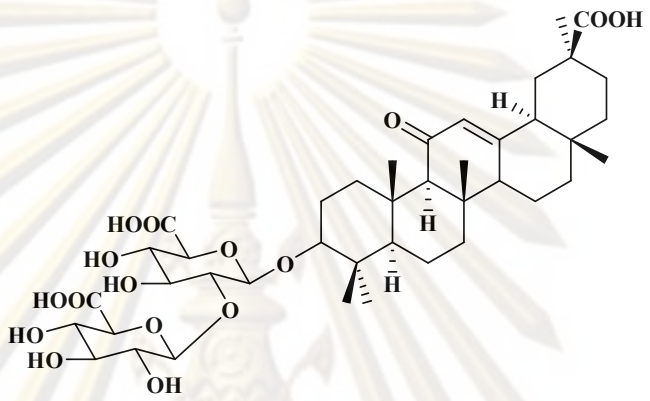

Figure 2.1 Chemical structures of glycyrrhizin in licorice (Yuan *et al.,* 2005)

2.2 Botanical aspects of *G. glabra*

Licorice is a member of the leguminosae which is one of the largest families of flowering plant on earth. The genus *Glycyrrhiza* consists of about 30 species in which *G.glabra*, *G. uralensis*, *G. inflata*, *G. aspera*, *G. korshinskyi* and *G. eurycarpa* are generally recognized as licorice because of their sweet taste. Two *Glycyrrhiza* species, *G. glabra* and *G. uralensis*, are known as major glycyrrhizin producing species, and their distribution in the world is different. *G. glabra* is found in Spain, Italy, Turkey, the Caucasus, Central Asia and the western part of China, whereas the distribution area of *G. uralensis* is from Central Asia to China. In addition to these two species, *G. inflata*, another GL-producing species, is found in the western part of China. Licorice is an herbaceous perennial, growing to 1 m in height, with pinnate leaves about 7–15 centimetres (3–6 inches) long, with 9–17 leaflets. The flowers are 0.8–1.2 cm (1/3 to 1/2 inch) long, purple to pale whitish blue, produced in a loose inflorescence. The fruit is an oblong pod, 2–3 centimetres (about 1 inch) long, containing several seeds (Figure 2.2).

Figure 2.2 Characteristics of *G. glabra.*

2.3 Chemical compositions of licorice (*G. glabra*) root

The chemical composition of licorice root has been reported by several researchers (Shibata and Zasshi 2000). Extensive chemical studies revealed that *Glycyrrhiza* plants produce not only glycyrrhizin but also many saponins and flavonoids (Shibata and Saitoh 1978). The variation of the contents of these saponins and flavonoids, from the phytochemical and biological point of view, can be attributed to environmental factors, such as geographical coordinates, altitude and solar exposure, harvesting and processing, and thus affect the therapeutic effects of licorice (Statti *et al.*, 2004). Among these variations, type of plant species and geographical location cause significant differences (Zhang and Ye 2009). For example,

2.3.1 Triterpene saponins

Triterpene saponins are the major characteristic constituents of licorice, which are responsible for the sweetening taste. Until now, at least 18 saponins have been obtained from the three *Glycyrrhiza* species. Most licorice saponins are present as glucuronides. The aglycones are oleanane type pentacyclic triterpenes with 11-oxo-12-ene, 12-ene, 11, 13(18)-diene or 9(11), 12-diene skeletons, and 3-OH, 24-OH, 22-acetoxy, 30-COOH or 29-COOH as functional groups (Sabbioni *et al*., 2005).

2.3.2 Flavonoids and other phenolic compounds

More than 300 flavonoids have been isolated from *Glycyrrhiza* species (Li *et al.,* 2000). These flavonoids belong to various types, including flavanones or flavanonols, chalcones, isoflavans, isoflavenes, flavones or flavonols, isoflavones and isoflavanones. Among them, flavanones and chalcones are of the major types. Aside from flavonoids, less popular phenolic compounds, like 3-aryl-coumarins coumestans (Lou and Qin 1995) and benzofuran (Fukai *et al.,* 1996), have also been obtained from *Glycyrrhiza* species. The phenolic compounds in licorice could be chemotaxonomic markers to differentiate *Glycyrrhiza* species, especially the three official species (*G. uralensis*, *G. glabra*, and *G.inflata*). Such main constituents as liquiritigenin, liquiritin, isoliquiritigenin and isoliquiritin are present in all the three official species. However, some compounds may be regarded as the species-specific components, which mean that they may only be present in specific species. For instance, glabridin and glabrene are only present in *G. glabra*, while glycycoumarin only in *G. uralensis*, and licochalcones A and B only in *G. inflate* (Hatano *et al.,* 1999)

2.4 Biosynthetic of Glycyrrhizin

Glycyrrhizin should be derived from a simple, *β*-amyrin (Figure 2.3), which is synthesized by *β*-amyrin synthase (bAS), an oxidosqualene cyclase (OSC). OSCs catalyze the cyclization of 2,3-oxidosqualene, a common intermediate of both triterpene and phytosterol biosyntheses (Abe *et al*. 1993; Haralampidis *et al*., 2002). In *G. glabra,* three OSCs: bAS, lupeol synthase (LUS) and cycloartenol synthase

(CAS) are situated at the branching step for biosynthesis of oleanane-type triterpene saponin, lupine type triterpene (betulinic acid) and phytosterols, respectively (Ayabe *et al.,* 1990; Hayashi *et al.,* 2004).

2.5 Biological activities

The pharmacological studies of licorice saponins focus on the main constituent glycyrrhizic acid and its aglycone glycyrrhetic acid. These two compounds exhibit extensive biological activities, including antiulceric, anti-inflammatory (Fujisawa *et al.,* 2000), antiallergic (Ram *et al.,* 2006), antioxidative (Yokosawa *et al.,* 2000), antiviral (Chen *et al.,* 2004), anticarcinogenic (Satomi *et al.,* 2005), antithrombotic (Mendes-Silva *et al.,* 2003), antidiabetic (Ko *et al.,* 2007), hepatoprotective (Shim *et al.,* 2000) neuroprotective (Cherng *et al.,* 2006) activities and others. Recently, glycyrrhizic acid has been used as a potential therapeutic agent for several virus diseases (Tandon *et al.,* 2002), including chronic hepatitis B and C, as well as human Acquired Immunodeficiency Syndrome (AIDS). In recent years, licorice flavonoids are gaining popularities because of their significant biological activities including antiulceric (Nakamura *et al.* 2003), antioxidative (Fukai *et al.,* 2003), anti-inflammatory (Furuhashi *et al.,* 2005), antimicrobial (Fukai *et al.,* 2002), antispasmodic (Sato *et al.,* 2007), antitumor (Yoon *et al.,* 2007), metabolic syndrome preventive (Tominaga *et al*., 2006) activities and others. Most of these activities are attributed to the major flavonoids, like liquiritigenin, liquiritin, isoliquiritigenin and isoliquiritin, as well assome unique compounds like glabridin, glabrene, licochalcone A, licochalcone B and glycycoumarin.

2.6 Plant tissue culture for regeneration (micropropagation)

Plant tissue culture techniques are standard procedures in modern biotechnology. This technique, known as micropropagation, is effective because almost all plant cells are totipotent. Each cell possesses the genetic information and cellular machinery necessary to generate an entire organism (Raven *et al*., 1999). Many important medicinal herbs have been successfully regenerated *in vitro* (Table 2.1). The advantages of this technology over the conventional agricultural production are as follows.

- propagation can be used to produce a large number of plants.
- cultures are genetically identical to the parent plant, as well as to one another.
- It is independent of geographical and seasonal variations and various environmental factors.
- explants are cultured individually under aseptic conditions on medium, free from pathogen.
- material can often be stored over a long period.

Murashige (1974) originally described three basic stages (I to III) for successful micropropagation. These stages have been used widely in many commercial and institutional tissue culture laboratories. Micropropagation stages for propagation by shoot culture are provided in Figure 2.4.

Stage I: Selection and preparation of explants

Plant which is a representative typical of each variety and disease free would be selected as a mother plant for *in vitro* culture. Growth, morphogenesis and rates of *in vitro* propagation can be improved by appropriate environmental and chemical pretreatment of mother plant.

Stage II: Development of aseptic culture

The second step in the micropropagation process is to obtain an aseptic culture of the selected plant material. The explants should be aseptically transferred to the culture environment and completed a number of survived explants without contamination.

Stage III: The production of suitable propagates

The object of this step is to bring about the multiplication of organs and structures that are able to give rise to new intact plants. This step includes the prior induction of meristematic centres from which adventitious organs may develop.

Stage IV: Preparation for growth in the natural environment

Shoots or plantlets derived from stage 3 are very small and not yet capable of self-supporting for growth in soil or compost. This step is taken to grow under individual plantlets that can carry out photosynthesis and survive without an artificial supply of carbohydrate. This step includes the *in vitro* rooting of shoots prior to their transfer to soil. In some species, this step is necessary to have elongated shoots ready for rooting.

Stage V: Transfer to the natural environment

This step is to transfer plantlets from the *in vitro* growth to the extra external environment carefully. This step is very important because improper transferring methods can result in a significant loss of new plants.

2.7 Plant tissue culture of licorice (*G. glabra*)

Recent development of *in vitro* technique has demonstrated its application in a rapid clonal propagation in short time, regeneration and multiplication selected superior clones and production of secondary metabolites. Depending on the species and culture conditions, the method that frequently used for commercial production is shoot tip culture method. Consequently, this method can be applied to micropropagation of *G. glabra.* To this end, Shah and Dalai (1980) reported *in vitro* multiplication of *G. glabra* on modified MS media. Syrtanova and Mukhitdinova (1984) attempted the tissue culture based multiplication of *G.glabra* and *G. uralensis* from seedling cultures. Although multiple shoot formation with some 90-95% plantlet survival upon transplanting into the field has been reported, they gave no experimental details as to how this was attained. Meanwhile, the development of callus and cell cultures from *G. glabra* has increasingly been reported and included from shoot tips, roots, leaves, stems and hypocotyl as explant tissue sources (Hayashi *et al*., 1988; Hayashi *et* al., 1992; Tamaki *et al*., 1973; Yoo and Kim, 1986). Moreover, this has included the production of *G. glabra* plants from shoot tip and nodal explant cultures on simple minimal media (Kohjyouma *et al.*, 1995; Thengane *et al.,* 1998). Regenerative callus formation, as well as cell suspensions, has also been reported recently (Mousa *et al*., 2007).

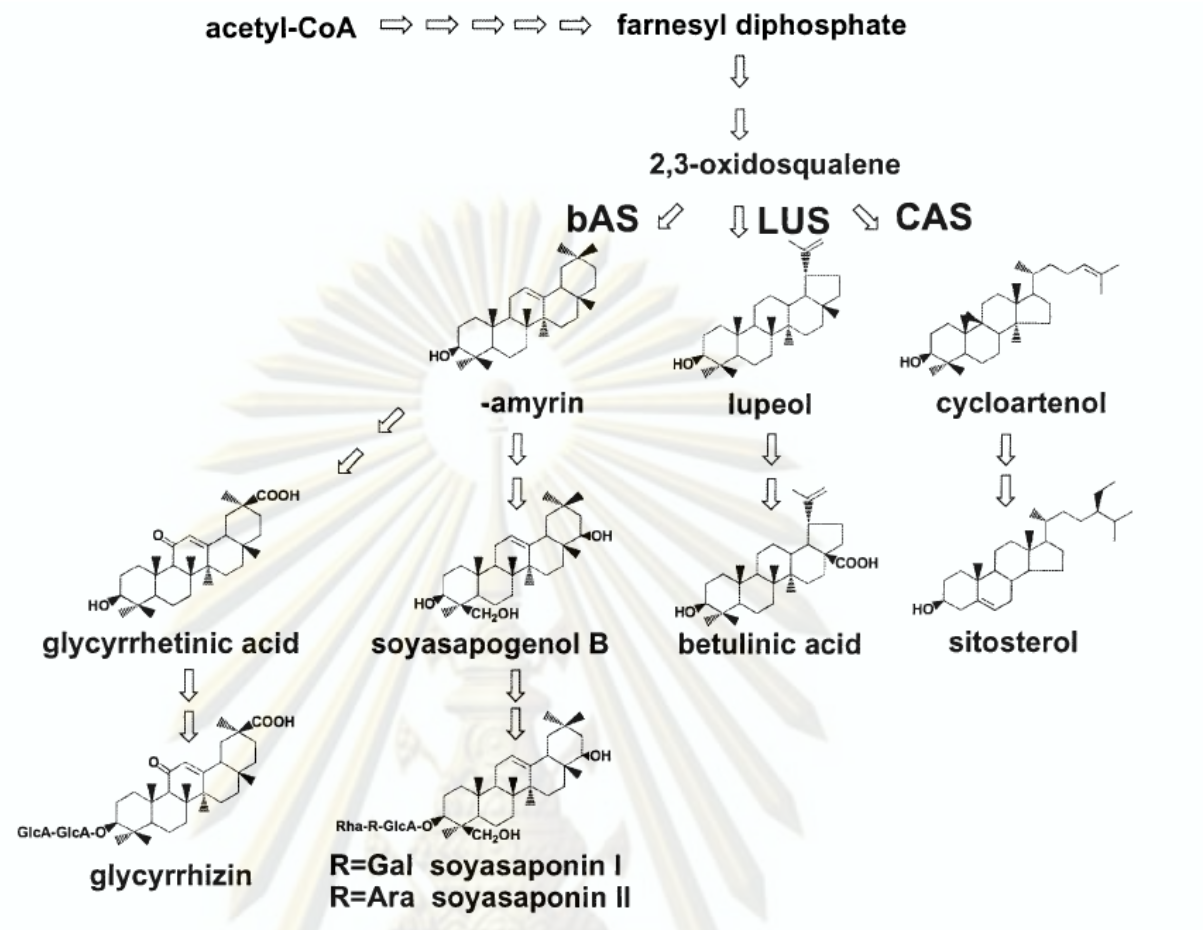

Figure 2.3 The biosynthetic pathways of triterpenoid and saponin in *G. glabra* (Hayashi *et al.,* 2004)

Figure 2.4 Micropropagation stages for production by shoot culture

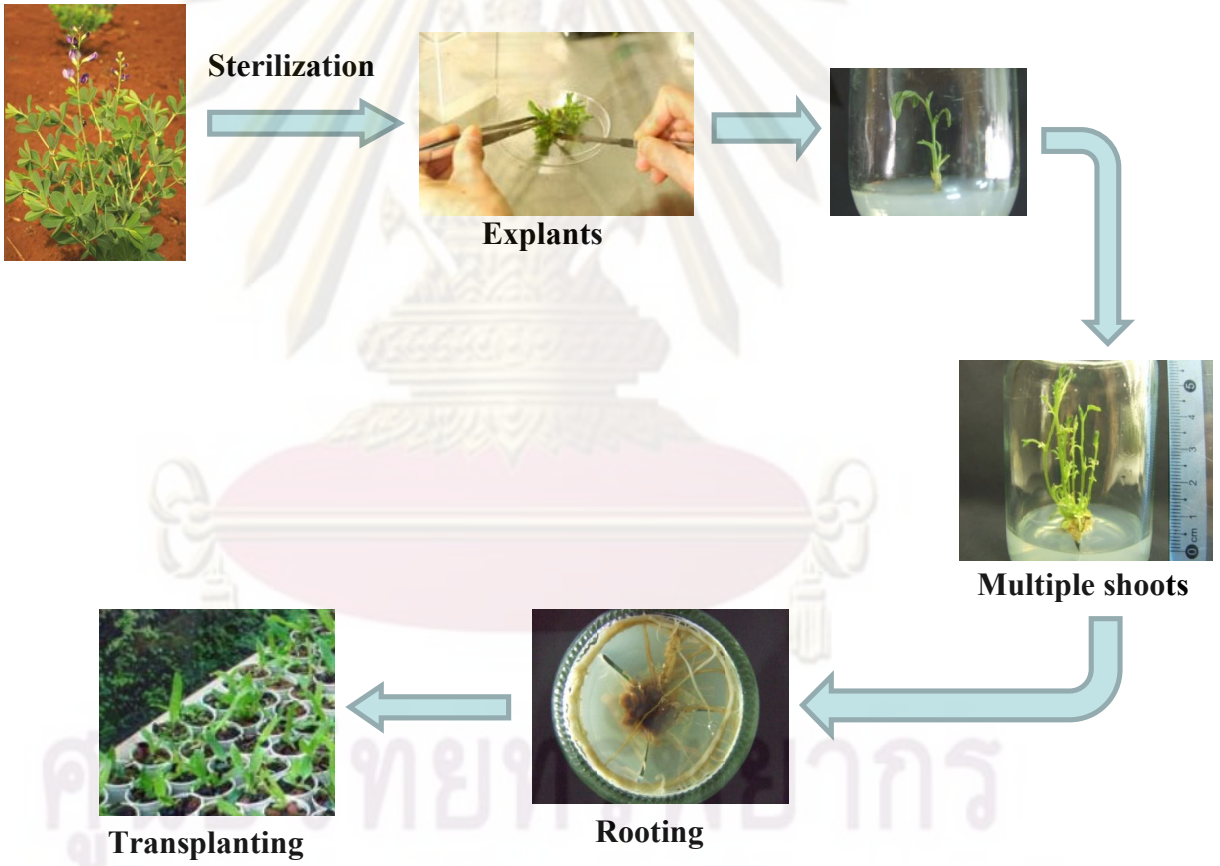

Table 2.1 *In vitro* studies of *Glycyrrhiza spp*.

	Plant species	Source of explants	Morphogenesis/propagation	References
	G. echinata	leaves, petioles	callus	Nakamura *et al.,* 1999
	G. glabra	axillary buds	multiple shoots	Kohjyouma *et al.,* 1995
		shoot tips, node	multiple shoots	Thengane *et al.,* 1998
	G. uralensis	cotyledons	callus	Kobayashi *et al.,* 1985

2.8 Glycyrrhizin formation in plant tissue culture

Many plants are major sources of natural products used as pharmaceuticals, agrochemicals, flavor, food additives, and pesticides. In the search for alternatives to production of medicinal compounds interest from plants, biotechnological approaches, specifically, plant tissue cultures, have potential as a supplement to traditional agriculture in the industrial production of bioactive secondary metabolites (Ramachandra Rao and Ravishankar, 2002) at a similar or superior value. According to the bioassay, it has been shown that the biosynthetic activity of cultured cells can be enhanced by plant growth regulators (Table 2.2).

Given that plant cell cultures are a promising alternative source for the production of valuable secondary metabolites; this potentially paves the way for potential glycyrrhizin production in tissue culture. However, despite the initial reports on the successful tissue culture production of licorice explants, the ability to produce glycyrrhizin in cell culture is controversial. Initial reports of its production by *Glycyrrhiza* sp. callus and suspension cultures, including within larger scale 80 L airlift bioreactors, at levels approaching 3-4% of the dry weight (Tamaki *et al*., 1973), were not replicated in callus or cell suspension cultures of a known *G. glabra* isolate (Hayashi *et al*., 1988). Wu *et al*. (1974) have reported the absence of glycyrrhizin in *G. glabra* var. typica in cell cultures, whereas Ko *et al*. (1989) have reported the production of glycyrrhizin in transformed hairy root cultures of *G. ulalensis*, they found no production in transformed roots of *G. glabra*.

Nevertheless, the formation of glycyrrhizin in *G. ulalensis* calli cultured in a Murashige & Skoog (MS) based medium supplemented with auxin (either naphthalene acetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D)) could be induced with specific combinations of the cytokinins benzyladenine (BA), 6-furfuryaminopurine (Kientin) or thiadiazuron (TDZ) (Shams-Ardakani *et al*., 2007; Wongwicha *et al*., 2008). Thus, in addition to variation in the genetic source (species and perhaps cultivars) of the tissue or cell suspension origins, the specific culture requirements including auxin/cytokinin concentrations or relative ratio, seem to be potentially important factors in glycyrrhizin production.

Table 2.2 Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures

Plant species	Active ingredient	Culture medium	Culture type	References
Buplerum falcatum L.	Saikosaponin	B5+IBA(8 mgL^{-1}), Sucrose(1-8%)	Root	Kusakari *et al.,* 2000
Eriobotrya japonica	Triterpenes	LS+NAA(10 μM), BA(10 μM)	Callus	Taniguchi *et al.,* 2000
Glycyrrhiza echinata	Flavonoids	MS+IAA(1 mgL^{-1}), Kinetin(0.1 mgL^{-1})	Callus	Ayabe *et al.,* 1986
Glycyrrhiza glabra var. *glandulifera*	Triterpenes	MS+IAA(5 ppm), or 2,4-D(1ppm), Kinetin (0.1pppm)	Callus	Ayabe *et al.,* 1990
Mentha arvensis	Terpenoid	MS+BA(5 mgL^{-1}), NAA(0.5 mgL^{-1})	Shoot	Phatak and Heble, 2002
Panax ginseng	Saponins and Sapogenins	MS(without Glysine)+ 2,4-D(1 mgL^{-1})	Callus	Furuya *et al.,* 1973
Polygola amarelle	Saponins	MS+IAA(1 mgL^{-1})	Callus	Desbene *et al.,* 1999

2.9 Objectives

1. To evaluate the optimal culture media and plant growth regulators on shoot proliferate and root induction for growth and development of *G. glabra.*

2. To evaluate the survival percentage of *G. glabra* during the acclimatization process using different culture substrates.

3. To determine time course for glycyrrhizin accumulation during culture and to enhance glycyrrhizin production using plant growth regulators.

CHAPTER III

MATERIALS AND METHODS

3.1 Plant materials

Shoots of *G. glabra* were taken from a single matured mother plant grew at the nursery of the Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Thailand.

3.2 Chemical agents

1. The culture media consisted of MS media (Murashige and Skoog, 1962), B5 media (Gamborg, 1968) and Woody plant media (Llyod & McCown, 1981) were tissue culture grade. The component of these media, compositions and preparation of stock solutions were described in the Appendix I, II.

2. Plant growth regulators used in this study were summarized as follow:

Auxins:

α -Napthaleneacetic acid (NAA), Fluka Biochemika, Switzerland

Indole-3-butylic acid (IBA), Fluka Biochemika, Swizerland

Indole-3-acetic acid (IAA), Sigma Chemical Co., USA

Cytokinins:

6-Benzyladenine (BA), Fluka Biochemika, Switzerland

Kinetin (Kn), Sigma Chemical Co., USA

Thidiazuron (TDZ), ChemService inc, USA

3. Chemical agents used in this study were included as follow:

Acetronitrile, HPLC grade from Merck

Methanol, HPLC grade from Merck

DDI Water

Standard Glycyrrhizic acid Ammonium salt, Fluka Biochemika, Switzerland

3.3 Instruments for analysis

1. HPLC instrument: ThermoFinningan HPLC SpectraSystem equipped with autosampler model AS3000, Photodiode array UV detector model UV6000LP and Chromquest Software.
2. HPLC column: Luna 5U C18(2) 100A (250×4.60 mm)
3. Rotary shaker
4. Laminar air flow model ZRE-330B, Yamato Co.,Ltd., Japan
5. Sonicator, D.S.C. Group Co.,Ltd., Thailand
6. Paper filter Whatman No.4
7. PTFE Microfilter size 0.45 μm
8. Micropipette 200, 1000 μl
9. Vial size 1.5 mL

3.4 Micropropagation of *G. glabra*

3.4.1 Plant sterilization

Shoot tip of *G. glabra* which 1.0-2.0 cm length were excised and rinsed for 30 min in running tap water. The undertrial material was surface sterilized for 15 min in 10% (v/v) Clorox containing 0.1% (v/v) Tween-20, and washed three times with sterile water under aseptic condition.

3.4.2 Initiation of cultures and culture conditions

Sterilized shoot explants were cultured on MS media containing 3.0% (w/v) sucrose as a carbon source and 0.7% (w/v) agar. The pH of medium was adjusted to 5.7 using 1N NaOH before autoclaving. The cultures were maintained at 25±2°C, 55 μmol $m^{-2}s^{-1}$ of 16 hrs. light with provided by a cool-white fluorescent tube. Subculturing was done every 4 weeks and served as a basal medium (MS). Depending on the experiment MS was also supplemented with various growth regulators.

3.4.3 Factors affecting of *G. glabra* plant tissue culture

Studying of various factors involved in propagation of *G. glabra* was carried out by the following methods:

1. The effect of medium formulations and concentration on shoot growth and development

To achieve a healthy plant from an explant, various kind of media (inorganic salt media) have been designed. One of the most commonly used media for plant tissue cultures is the MS medium that developed by Murashige and Skoog. The significant feature of the MS medium is its very high concentration of nitrate, potassium and ammonia. The B5 medium established by Gamborg is also being used by many researchers. The levels of inorganic nutrients in the B5 medium are lower than in MS medium. Other media that are commonly used include Lloyd and McCown's Woody Plant Medium (WPM) that has been widely used successfully for a great many tree species. In order to obtain higher productivity and growth rate, the most suitable medium composition should be optimized.

Shoot explants from 4 weeks were cut transversely and cultured on different media formulation with three types of basal media; MS (Murashige & Skoog 1962), B5 (Gamborg 1968) and WPM (Llyod and McCown 1981). Each media vary strength of salt base including 1-, 1/2-, 1/4-strength concentrations containing 3.0% (w/v) sucrose and 0.7% (w/v) agar. The pH of medium was adjusted to 5.7; these cultures were grown under condition stated above. The number and length of the shoots, the stem diameter and leaf width were all recorded after 6 weeks of culture.

2. The effect of sucrose concentration on shoot growth and development

Sucrose is suitable carbon sources which is added to the basal medium**.** However, the most suitable carbon source and its optimal concentration should be chosen to establish the efficient production process of useful metabolites. These factors depend on plant species and them products therefore it is necessary to optimize the medium compositions including carbon sources in each case.

The excised shoots from 4 weeks were placed on medium containing different concentration of sucrose (0, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0% (w/v)). The cultures were maintained at 25±2°C with 16 hrs. photoperiod. The average number and length of shoots, the stem diameter and leaf width were all recorded after 6 weeks of culture.

3. The effect of plant growth regulators on shoot growth and development

Plant growth regulators required to induce shoot multiplication and rooting. Cytokinins and Auxins are the most widely used plant growth regulators in plant tissue culture. During multiplication phases, cytokinins such as BA are used to achieve multi-shoot plants, whereas auxins such as IBA are used to induce root formation on culture shoots. Since each plant species requires different kinds and levels of plant growth regulators for shoots and roots induction, therefore it is important to select the most appropriate growth regulators and to determine their optimal concentrations.

A. The effect of cytokinin types on shoot multiplication

Shoot explants after 4 weeks culture were cut transversely and transfer to the suitable medium (determined from the results of the above trails) supplemented with 3.0% (w/v) sucrose and 0.7% (w/v) agar containing with different concentrations of the cytokinins (0, 0.5, 1.0, 1.5, 2.0 and 2.5 mgL^{-1}) of one of benzyladenine (BA), 6- Furfuryaminopurine (Kientin) or thiadiazuron (TDZ). The cultures were maintained under condition as described in the previous section for six weeks. The number and length of shoots per plantlet were measured.

B. The effect of auxins types on rooting ability

To optimize root induction, *in vitro* shoot explants after four weeks of culture were excised and transferred individually to medium supplemented with 3.0% (w/v) sucrose and 0.7% (w/v) agar containing different concentrations of

auxins (0, 1.0, 2.0, 3.0, 4.0 and 5.0 mgL^{-1}) of one of α -Napthaleneacetic acid (NAA), Indole-3-acetic acid (IAA) or Indole-3-butylic acid (IBA). The excised shoot explants were cultured for six weeks and then the average length of the roots, roots diameter and roots weight were measured.

3.5 Acclimatization of plantlets

Plantlets having well developed root were removed from the culture and washed free of agar media and subsequently transferred individually to plastic bags containing with SC1, sterile garden soil; SC2, vermiculite; SC3, garden soil mixed with vermiculite and ash husk (1:1:1); SC4, ash husk; SC5, garden soil mixed with ash husk (1:1). These pots were covered with transparent plastic cover to ensure high humidity and kept at room temperature in natural light for about a week. Acclimatized plants were then transferred to the greenhouse. The percentage of survival was observed.

3.6. Quantitative analysis of Glycyrrhizin from *G. glabra*

3.6.1 Preparation of plant materials

Sterilized shoot explants were cultured individually under aseptic conditions on MS media containing 3.0% (w/v) sucrose as a carbon source. The pH of medium was adjusted to 5.7 using 1N NaOH and 1N HCl before gelling with agar (0.7% w/v) and 15 ml of medium were dispensed in 4 oz. bottles and autoclaved at 1.5 atm of pressure, 121°C for 15 min. The cultures were maintained at 25±2°C in the presence of 16/8 hrs. photoperiod.

3.6.2 Factors involved glycyrrhizin formation in plant tissue cultures

1. Growth stages

For the time course study, untreated plants and those supplemented with plant growth regulators in the root culture media were harvested at 6, 8, 10 and 12 weeks. To observe the effect of root age in response to plant growth regulators,

roots in different growth stages were treated with a specific amount of plant growth regulator. Glycyrrhizin content refers to the total amount of glycyrrhizin recovered from 0.1 g dry weight of root powder. The method for extraction of glycyrrhizin contents were described in section 6.

2. Plant growth regulators

Plant growth regulator concentration is often a crucial factor in secondary metabolites accumulation (DiCosmo and Towers, 1984; Deus and Zenk, 1982). The type and concentration of Auxin or Cytokinin or the Auxin/Cytokinin ratio alters dramatically causes both the growth and the product formation in cultured plant cells (Mantell and Smith, 1984).

Shoot cultures from 6 weeks old were cut and transferred to half-strength B5 medium. Each medium was supplemented with constant concentration (5.0 mgL^{-1}) of auxins (IAA and IBA). Roots were harvested and analyzed at different time intervals. Three replicates of samples were maintained for each treatment conditions. The method for extraction of glycyrrhizin contents were described in section 6.

3.6.3 Sample preparation for HPLC analysis

1. Preparation of standard solutions

Pure glycyrrhizin (Fluka, Catalog no. 2588877, lot no.1354108) was used as standard solution. A 10 mg of glycyrrhizin standard were accurately weighed and placed in 10 ml volumetric flask. Deionized water was used as the solvent to give a 1 mg/ml mixed standard solution. The standard solutions were made up to volume with deionized water to give a series of standard solutions of 0.00, 0.025, 0.05, 0.1, 0.25, 0.5 and 1.0 mg/ml, used for the calibration curve.

2. Preparation of sample solutions

The roots of licorice were dried at 50 °C to constant weight. A 100 mg of dried sample was pulverized and then extracted with 20 ml of 95% methanol

solution for 12 hrs. before filtering and evaporating the filtrate to dryness to attain a crude glycyrrhizin preparation and proceeded as schematic shown in Figure 3.1. The sample solution was prepared by dissolving the crude extract in 1 ml of 95% methanol solution and then a 10 µl aliquot of this extract solution were analyzed by HPLC.

3. High performance liquid chromatography conditions

Glycyrrhizin extract in all treatments of *Glycyrrhiza* were analyzed by HPLC. The conditions of HPLC analysis were described below:

HPLC conditions:

HPLC Column	:	Phenomenex column (Luna 5U C18(2) 100A, size 250×4.60 mm)
Mobile phase	:	Acetonitrile/Water (1% AcOH)(Gradient elution from 0 min (10:90), 15 min (50:50), 40 min (60:40) and 50 min (100:0)
Flow rate	:	0.9 ml/min for 40 min
Injection volume	:	10 µl
Wavelength Detection	:	254 nm

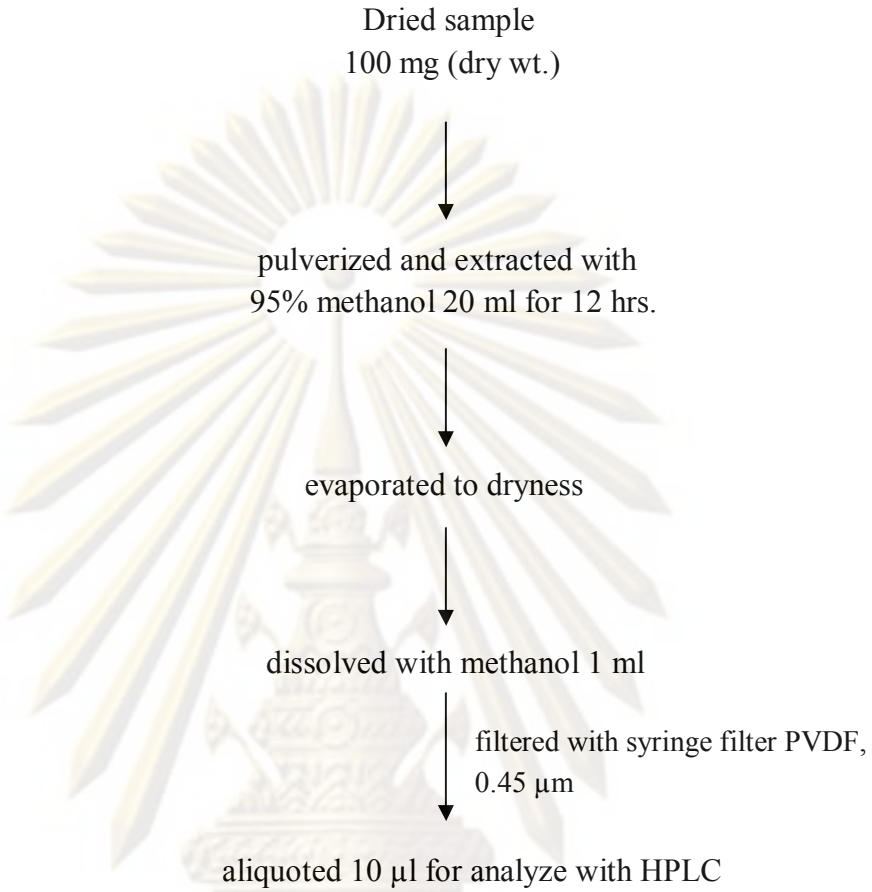

Figure 3.1 Sample preparation for HPLC analysis

3.7 Statistic analysis

A complete randomized design (CRD) was used for all experiments. The data were analyzed using SPSS Version 16.0. ANOVA was used to analyze data. Significant differences were assessed using Scheffe's multiple range test and significance was accepted at $P < 0.05$.

CHAPTER IV

RESULTS

4.1 Tissue culture of *G. glabra*.

4.1.1 Effect of medium formulations and its concentration on shoots growth and development

To optimize the culture medium on shoots growth and development, shoot tips of *in vitro* grown plantlets after 1 month of culture on MS medium were used as the starting materials. The morphogenesis responses in each treatment were observed according to Table 4.1. It has been shown that 1/2-strength B5 salt was suitable for *G. glabra* plants growth when observe morphogenic potentialities (i.e. shoot length, stem diameter, leaf width and number of branches) of other medium. 1/2-strength B5 salt yielded the maximum value in shoot length, leaf width and number of branches and significantly different as compare to full strength B5 salt and 1/4 strength B5 salt respectively. In addition, significant occurs in stem diameter and leaf width when comparison between medium types.

Meanwhile, 1/4-strength MS salt resulting healthily shoot, uniform stem, width leaves and strong branches as compared to those grown on the same medium (Figure 4.3, 4.4 and 4.5). Therefore, sugar concentrations will be the major key in next experiments on 1/2-strength B5 and 1/4-strength MS medium. However a magnificent effect on reduction of medium had the maximum value about 1.5 in number of shoots was found on full-strength MS salts (Figure 4.1). Therefore, further experiments were conducted to assess the increases in shoot multiplication rate with cytokinins at different concentration with full-strength MS salts.

Table 4.1 Effect of different media and its concentrations on shoot growth and development in *G. glabra* after culturing for 6 weeks.

Medium	Strength	Number of shoots/explant	Shoot length (cm)	Stem diameter (cm)	Leaf width (cm)	Number of branches/explant
B5	1	1.40 ± 0.15^{A}	5.00 ± 0.27^{B}	0.07 ± 0.00^{A}	0.60 ± 0.02^{AB}	6.30 ± 0.26^{B}
	1/2	1.25 ± 0.12^{AB}	6.58 ± 0.32^{A}	0.06 ± 0.00^{A}	0.68 ± 0.03^{A}	8.00 ± 0.33^{A}
	1/4	1.00 ± 0.00^{B}	5.94 ± 0.38^{AB}	0.05 ± 0.00^{B}	0.54 ± 0.03^{B}	7.35 ± 0.25^{A}
MS	1	1.50 ± 0.20^{A}	5.45 ± 0.40^{A}	0.05 ± 0.03^{A}	0.36 ± 0.02^{B}	4.95 ± 0.21^{B}
	1/2	1.45 ± 0.21^{A}	5.80 ± 0.42^{A}	0.05 ± 0.02^{A}	0.32 ± 0.03^{B}	5.95 ± 0.35^{AB}
	1/4	1.10 ± 0.07^{A}	5.00 ± 0.30^{A}	0.06 ± 0.02^{A}	0.54 ± 0.02^{A}	6.35 ± 0.22^{A}
WPM	1	1.00 ± 0.00^{A}	5.12 ± 0.35^{B}	0.06 ± 0.00^{A}	0.43 ± 0.03^{AB}	3.55 ± 0.48^{B}
	1/2	1.05 ± 0.05^{A}	6.30 ± 0.30^{AB}	0.05 ± 0.00^{B}	0.49 ± 0.02^{A}	4.85 ± 0.36^{AB}
	1/4	1.00 ± 0.00^{A}	6.48 ± 0.35^{A}	0.04 ± 0.00^{C}	0.38 ± 0.02^{B}	5.90 ± 0.24^{A}

Data represent mean value of 20 culture ±S.E. Within a column, values with different superscript letters are significantly different ($P < 0.05$) using Scheffe's multiple range test.

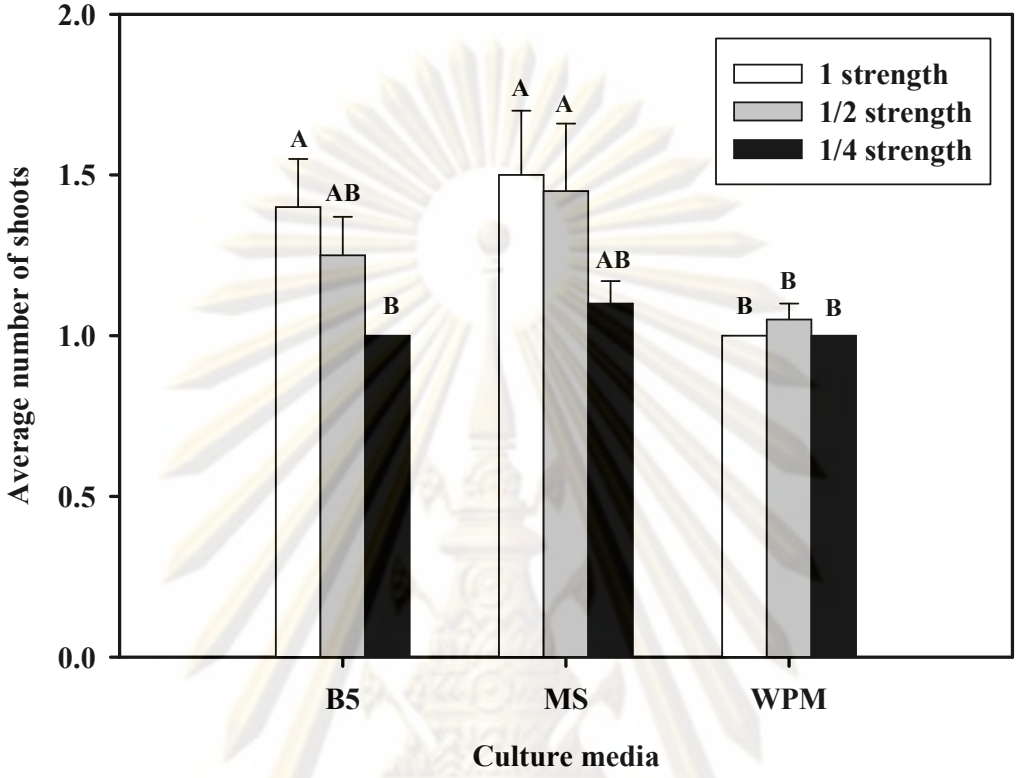

Figure 4.1 Effect of medium types on average number of shoots of *G. glabra* after 6 weeks. Each bar represents the mean ±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B was determined by Scheffe's multiple range test.

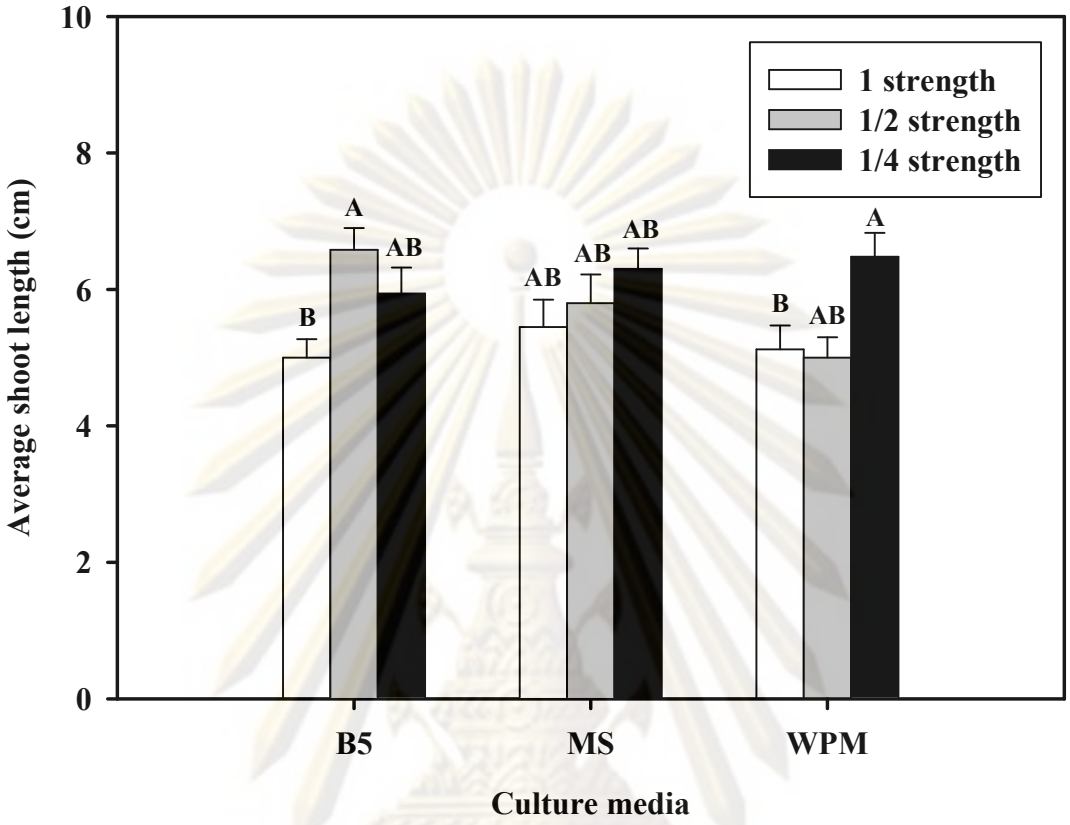

Figure 4.2 Effect of medium types on average shoot length of *G. glabra* after 6 weeks. Each bar represents the mean ±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B was determined by Scheffe's multiple range test.

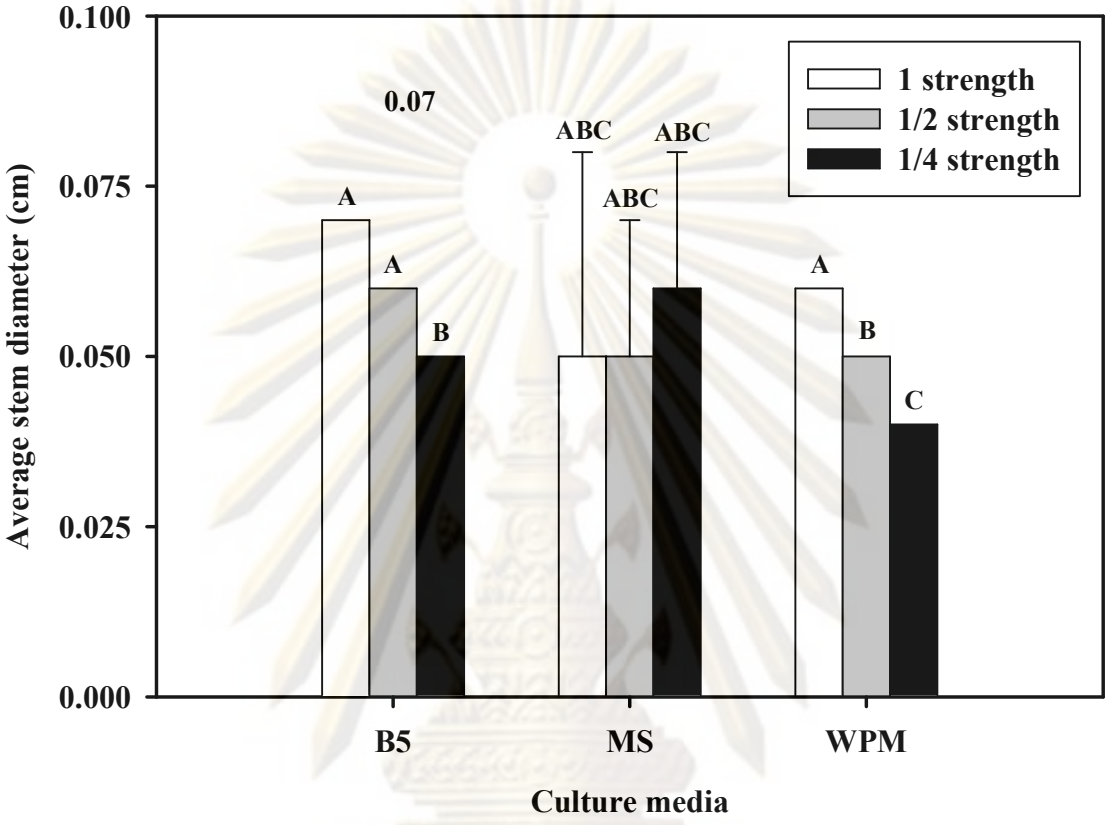

Figure 4.3 Effect of medium types on average stem diameter of *G. glabra* after 6 weeks. Each bar represents the mean ±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B, C was determined by Scheffe's multiple range test.

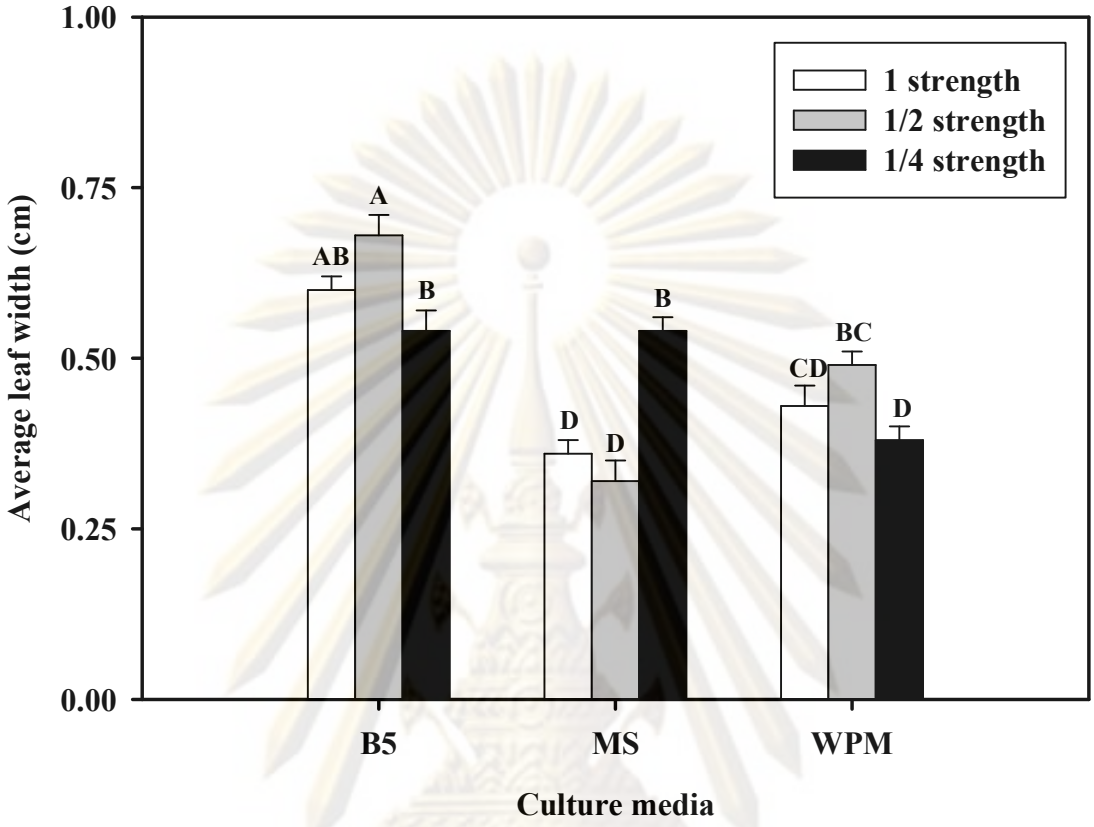

Figure 4.4 Effect of medium types on average leaf width of *G. glabra* after 6 weeks. Each bar represents the mean ±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B, C, D was determined by Scheffe's multiple range test.

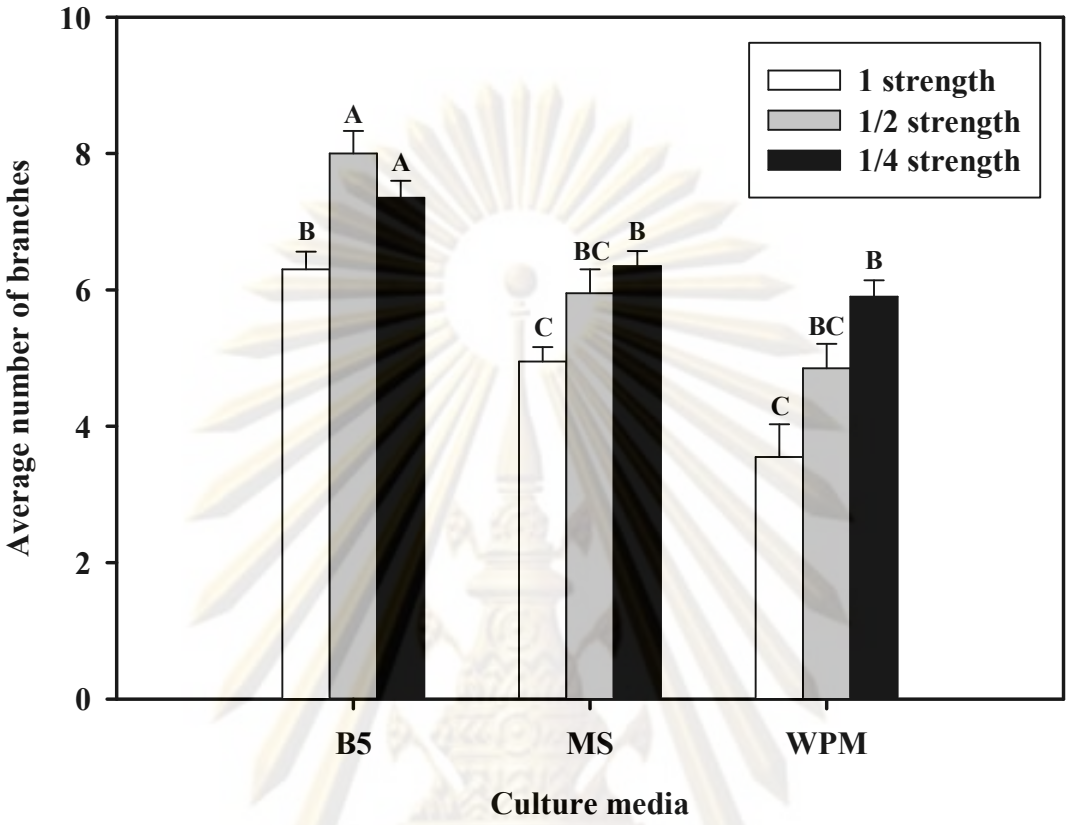

Figure 4.5 Effect of medium types on average number of branches of *G. glabra* after 6 weeks.Each bar represents the mean±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B, C was determined by Scheffe's multiple range test.

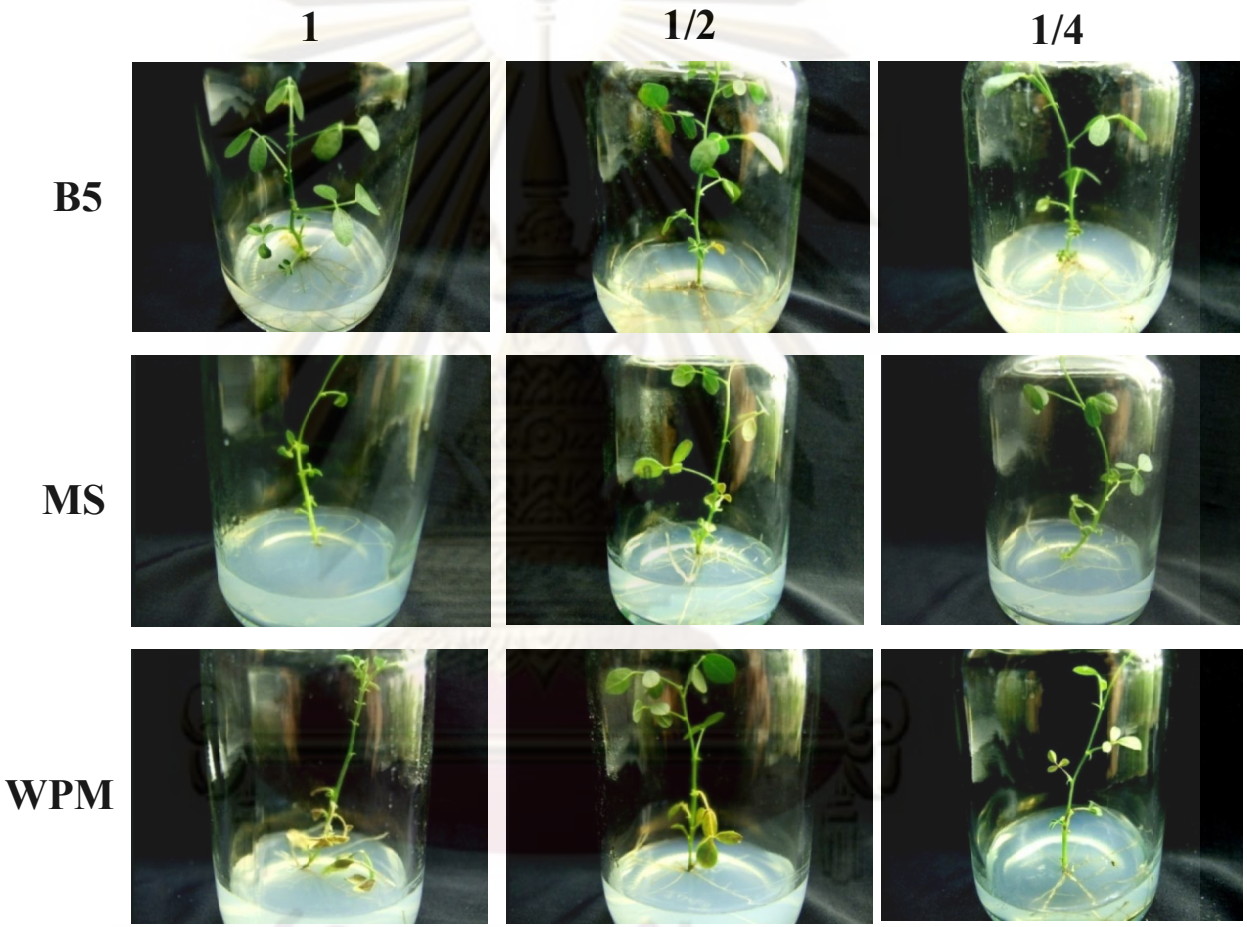

Figure 4.6 Shoot explants of licorice on various medium with different of salt base observed after 6 weeks.

4.1.2 Effect of sucrose concentrations on shoots growth and development

The effect of sucrose concentration on shoots growth and development was also studied. Various concentrations of sucrose gave different shoot growth and development. The characteristics of shoot growth were shown in Table 3.2. Sugars serve as the energy source for plant cultures. Because of imitations in CO_2 availability and plant tissue require enough sugar to add to media for growth. The results of shoot length and number of branches from the explants cultured on 1/4-strength MS medium gave a higher value when compared with 1/2-strength B5 medium at 10 and 30 gL^{-1} sucrose concentration (Figure 4.7 and 4.10).

On other hand, the results of stem diameter and leaf width from the explants cultured on 1/2-strength B5 medium gave a higher value when compared with 1/4-strength MS medium at sucrose concentration of ranging from 30-60 gL^{-1} (Figure 4.8 and 4.9). Therefore, the high efficiency of shoot growth that promoted shoot length, stem diameter, leaf width and number of branches was obtained at 30 gL^{-1} sucrose.

Table 4.2 Effect of sucrose concentration on shoots growth and development from shoot explant of *G. glabra* after culturing for 6 weeks

Medium	Sugar concentration (gL^{-1})	Shoot length (cm)	Stem diameter (cm)	Leaf width (cm)	Number of branches/explant
1/2B5	0	2.67 ± 0.29^{B}	0.04 ± 0.00^{A}	0.18 ± 0.02^{B}	4.80 ± 0.25^{A}
	10	5.45 ± 0.46^{A}	0.04 ± 0.00^{A}	0.26 ± 0.03^{AB}	5.25 ± 0.33^{A}
	20	5.44 ± 0.54^{A}	0.05 ± 0.00^{A}	0.34 ± 0.02^{A}	5.10 ± 0.28^{A}
	30	3.76 ± 0.37^{AB}	0.06 ± 0.02^{A}	0.32 ± 0.04^{AB}	4.86 ± 0.70^{A}
	40	4.13 ± 0.59^{AB}	0.06 ± 0.00^{A}	0.32 ± 0.05^{AB}	5.60 ± 0.31^{A}
	50	2.82 ± 0.57^{AB}	0.05 ± 0.00^{A}	0.21 ± 0.04^{AB}	5.00 ± 0.45^{A}
	60	2.57 ± 0.33^{B}	0.06 ± 0.00^{A}	0.25 ± 0.03^{AB}	5.00 ± 0.43^{A}
1/4MS	0	1.90 ± 0.22^{C}	0.06 ± 0.00^{AB}	0.07 ± 0.01^{B}	3.31 ± 0.38^{C}
	10	7.50 ± 0.59^{A}	0.04 ± 0.00^{B}	0.22 ± 0.02^{A}	6.42 ± 0.30^{AB}
	20	5.46 ± 0.56^{A}	0.05 ± 0.00^{AB}	0.24 ± 0.02^{A}	7.20 ± 0.40^{A}
	30	6.32 ± 0.56^{A}	0.05 ± 0.00^{A}	0.24 ± 0.02^{A}	6.95 ± 0.32^{AB}
	40	4.24 ± 0.56^{B}	0.05 ± 0.00^{A}	0.25 ± 0.02^{A}	5.10 ± 0.35^{BC}
	50	4.68 ± 0.60^{B}	0.06 ± 0.00^{A}	0.25 ± 0.03^{A}	5.32 ± 0.35^{B}
	60	4.21 ± 0.47^{B}	0.05 ± 0.00^{A}	0.18 ± 0.02^{A}	5.21 ± 0.48^{BC}

Data represent mean value of 20 culture± S.E. Within a column, values with different superscript letters are significantly different ($P < 0.05$) using Scheffe's multiple range test.

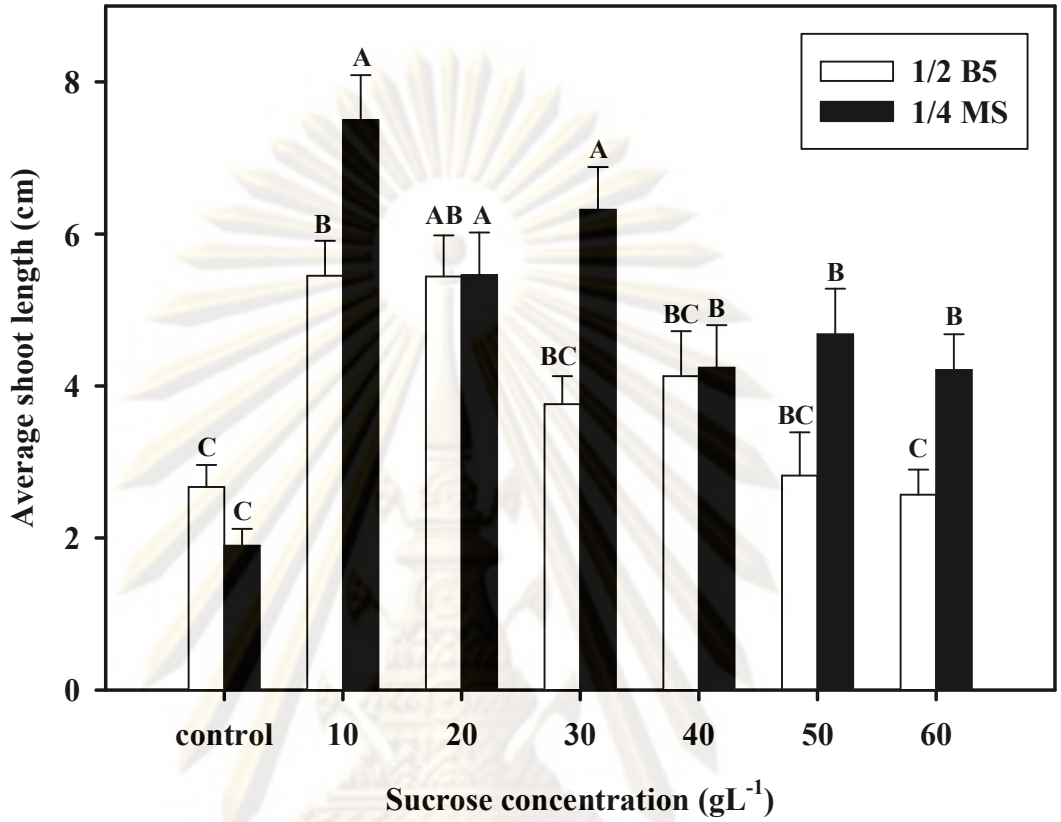

Figure 4.7 Effect of sucrose concentrations on average shoot length of *G. glabra* after 6 weeks. Each bar represents the mean±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B, C was determined by Scheffe's multiple range test.

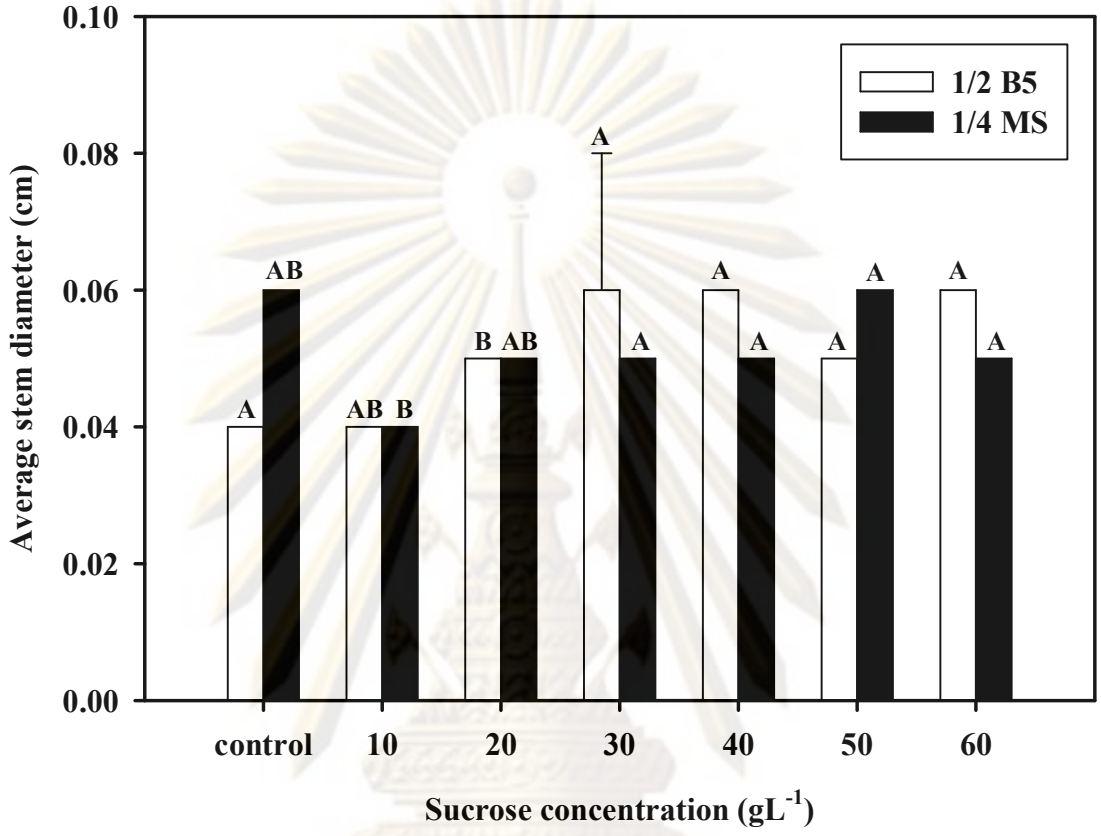

Figure 4.8 Effect of sucrose concentrations on average stem diameter of *G. glabra* after 6 weeks. Each bar represents the mean±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B was determined by Scheffe's multiple range test.

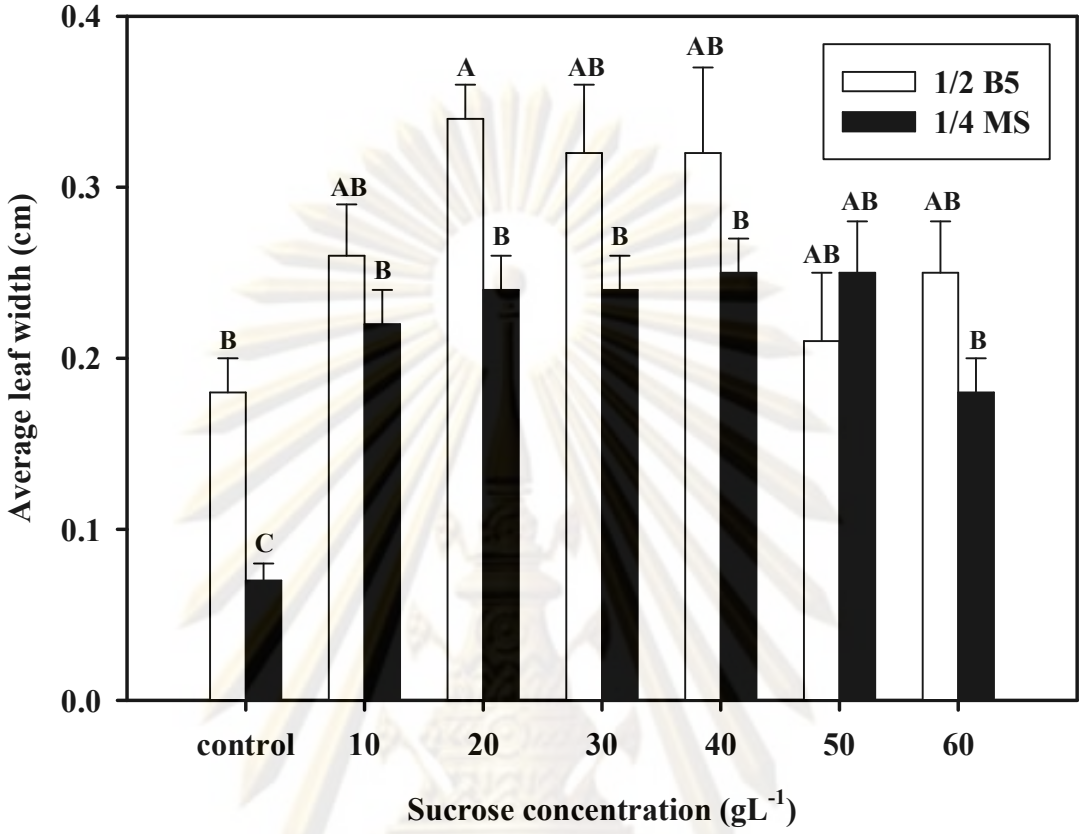

Figure 4.9 Effect of sucrose concentrations on average leaf width of *G. glabra* after 6 weeks. Each bar represents the mean±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B, C was determined by Scheffe's multiple range test.

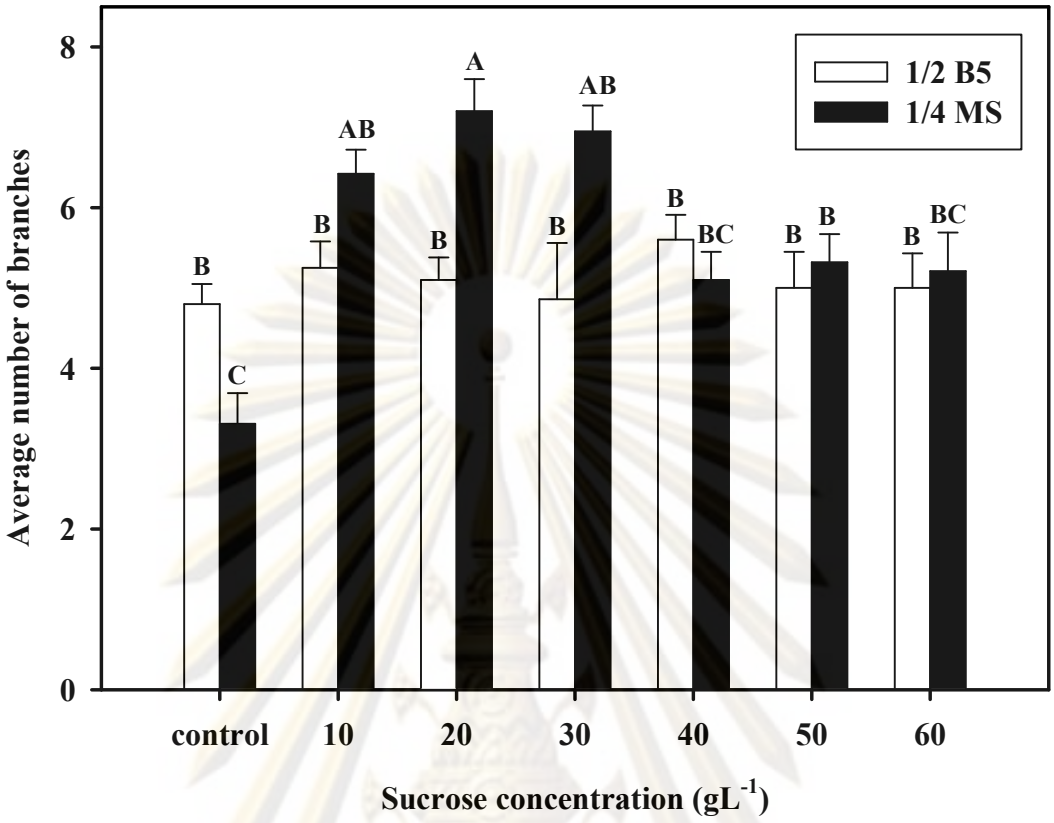

Figure 4.10 Effect of sucrose concentrations on average number of branches of *G. glabra* after 6 weeks. Each bar represents the mean ±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B, C was determined by Scheffe's multiple range test.

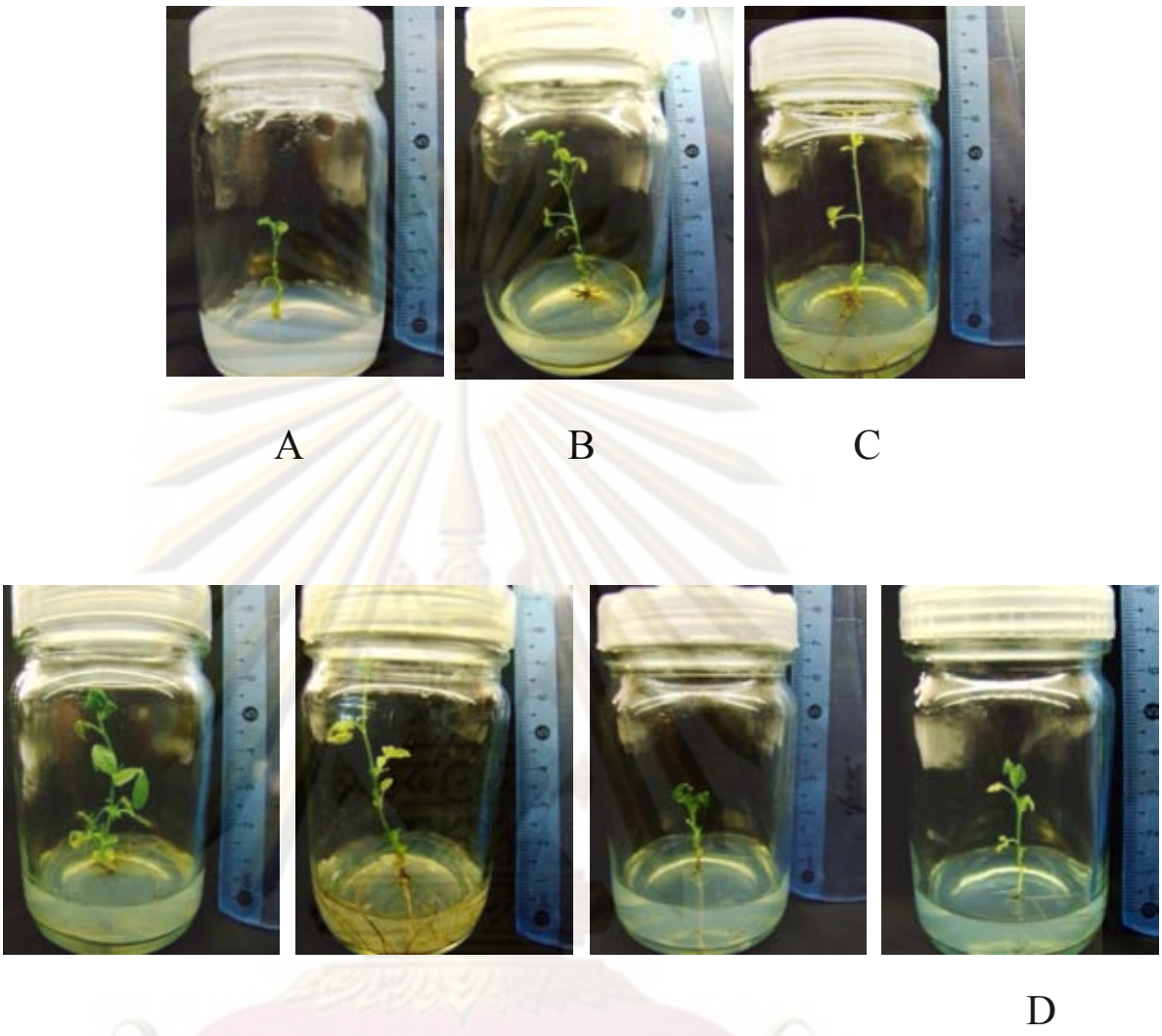

Figure 4.11 Characteristics of shoots growth cultured on 1/2 strength B5 medium supplemented with various concentrations of sucrose for 6 weeks. sucrose concentration; (A) 0 gL^{-1}, (B) 10 gL^{-1},(C) 20 gL^{-1}, (D) 30 gL^{-1} , (E) 40 gL^{1}, (F) - 50 gL^{-1}, (G) 60 gL^{-1}

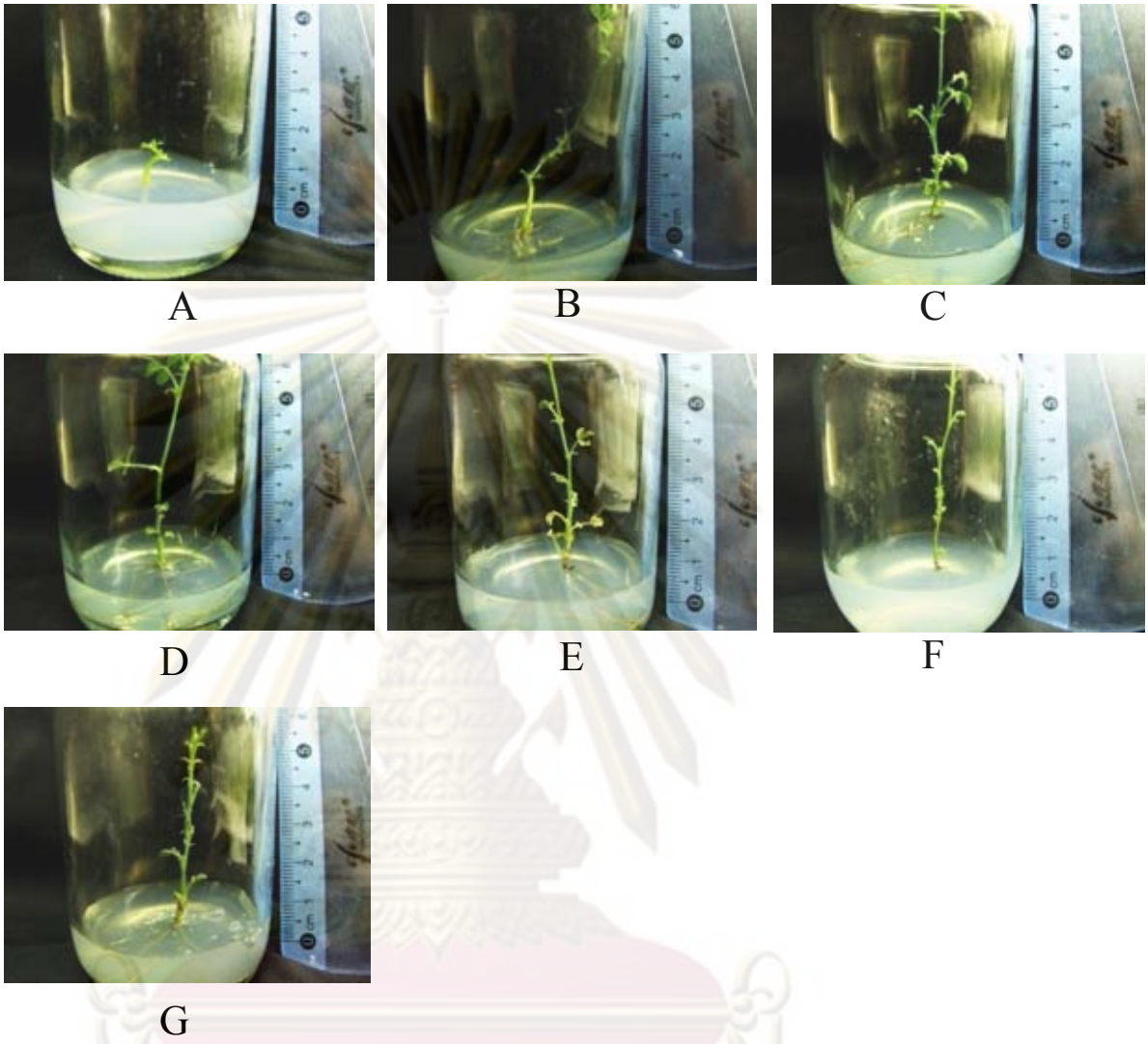

Figure4.12 Characteristics of shoots growth cultured on 1/4 strength MS medium supplemented with various concentrations of sucrose for 6 weeks. sucrose concentration; (A) 0 gL^{-1} , (B) 10 gL^{-1},(C) 20 gL^{-1}, (D) 30 gL^{-1} , (E) 40 gL^{-1}, (F) - 50 gL^{-1}, (G) 60 gL^{-1}

4.1.3 Effect of cytokinin on shoot multiplication

The influence of cytokinins on shoot multiplication was shown in Table 4.3. Observations, expressed as average number of shoot and length of shoot, were recorded after 6 weeks. The concentration of three types of cytokinins which were BA, TDZ and kinetin was supplemented at concentration of 0, 0.5, 1.0, 1.5, 2.0 and 2.5 $mg.L^{-1}$ in MS medium. In the present protocol, shoot cultured on MS medium containing 0.5 mgL^{-1} BA after cultured for 6 weeks proved to be the most effective as on this medium not only the number of shoots gave a higher number of shoots, 4.75 per explants but also, the shoot length was the highest (Figure 4.13, 4.14 and 4.15).

Table 4.3 Effect of cytokinin on shoot induction from *G. glabra* shoot explants, cultured on MS medium.

Cytokinins concentrations (mgL^{-1})			Number of shoots	Shoot length (cm)
BA	TDZ	Kinetin		
0	0	0	1.10 ± 0.10^{C}	5.97 ± 0.39^{A}
0.5	0	0	4.75 ± 0.42^{A}	3.78 ± 0.33^{B}
1.0	0	0	2.70 ± 0.46^{B}	3.57 ± 0.40^{BC}
1.5	0	0	1.25 ± 0.12^{BC}	2.22 ± 0.22^{CD}
2.0	0	0	1.25 ± 0.12^{BC}	1.98 ± 0.18^{D}
2.5	0	0	1.40 ± 0.13^{BC}	2.26 ± 0.20^{CD}
0	0	0	1.10 ± 0.07^{A}	4.26 ± 0.20^{A}
0	0.5	0	1.85 ± 0.43^{A}	1.08 ± 0.06^{B}
0	1.0	0	2.40 ± 0.44^{A}	0.98 ± 0.03^{B}
0	1.5	0	1.00 ± 0.00^{A}	1.11 ± 0.06^{B}
0	2.0	0	1.05 ± 0.05^{A}	1.01 ± 0.08^{B}
0	2.5	0	1.15 ± 0.11^{A}	1.26 ± 0.11^{B}
0	0	0	1.20 ± 0.09^{A}	5.00 ± 0.44^{A}
0	0	0.5	1.10 ± 0.07^{A}	3.13 ± 0.30^{B}
0	0	1.0	1.00 ± 0.0^{A}	2.39 ± 0.19^{B}
0	0	1.5	1.35 ± 0.13^{A}	2.07 ± 0.22^{B}
0	0	2.0	1.40 ± 0.18^{A}	2.24 ± 0.24^{B}
0	0	2.5	1.70 ± 0.25^{A}	3.13 ± 0.40^{B}

Data represent mean value of 20 culture ±S.E. Within a column, values with different superscript letters are significantly different ($P < 0.05$) using Scheffe's multiple range test.

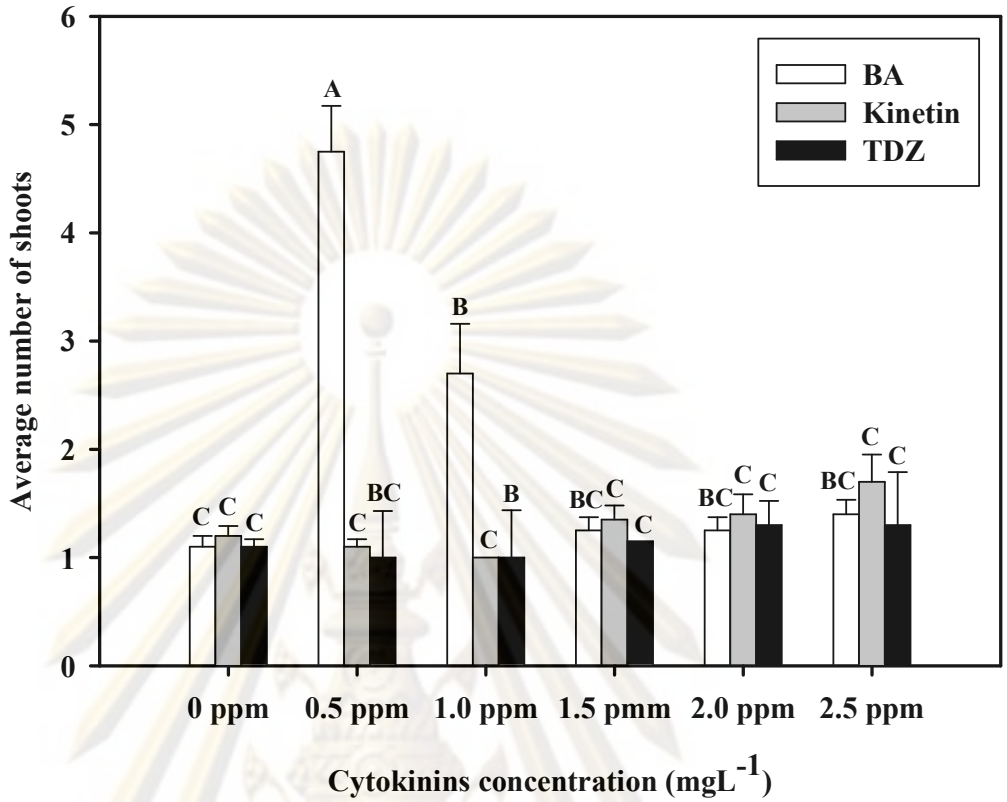

Figure 4.13 Effect of cytokinins on average shoots number of *G. glabra* after cultured on MS medium for 6 weeks. Each bar represents the mean ±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B, C was determined by Scheffe's multiple range test.

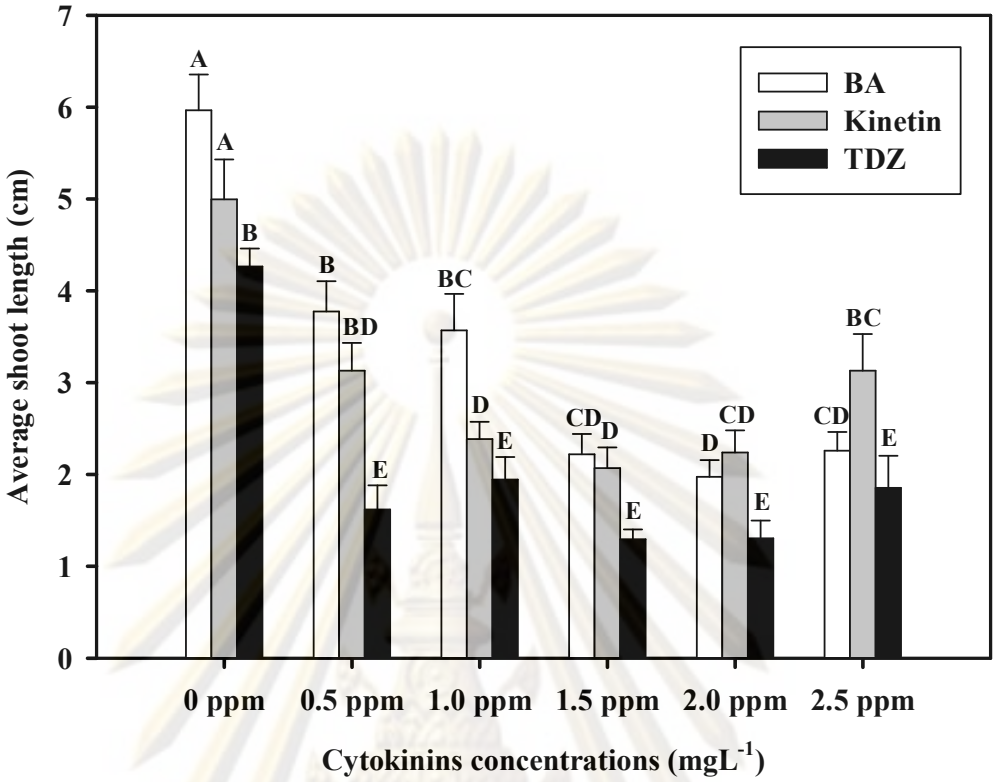

Figure 4.14 Effect of cytokinins on average shoots length of *G. glabra* after cultured on MS medium for 6 weeks. Each bar represents the mean ±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B, C, D, E was determined by Scheffe's multiple range test.

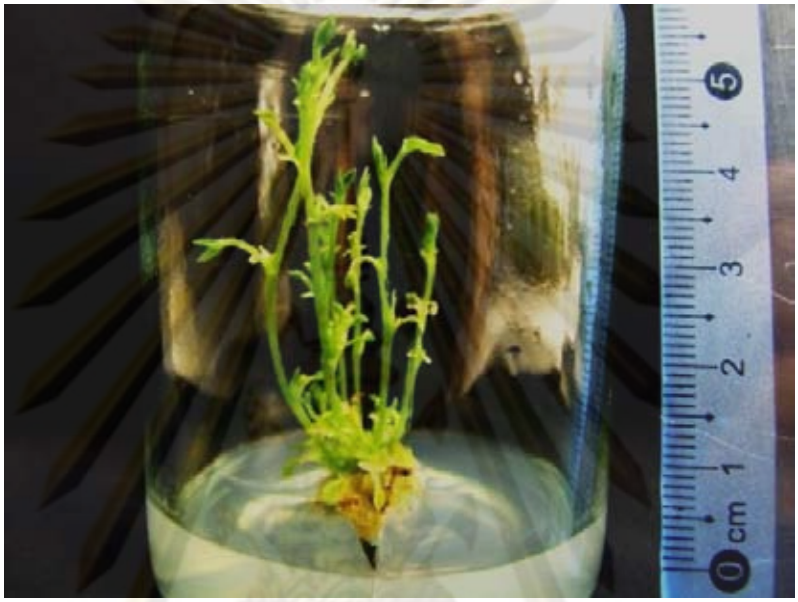

Figure 4.15 Multiple shoot from shoot tip of *G. glabra* after cultured 6 weeks on MS medium supplemented with 0.5 mgL^{-1} BA.

4.1.4 Effect of auxin types on rooting ability

The concentration of three types of auxins which were IAA, IBA and NAA was supplemented at 0, 1.0, 2.0, 3.0, 4.0 and 5.0 $mg.L^{-1}$ in 1/2-strength B5 medium. The influence of auxins on root induction was shown in Table 4.4. Observations, expressed as percentage of root induction and average length of root, were recorded after 6 weeks. In this experiment, it was found that the high percentage of root formation was obtained on the medium supplemented with 5.0 mgL^{-1} of either IAA or IBA (Table 4.5). However, whilst the addition of IBA in the rooting medium for explants was found to be more suitable for root induction than IAA containing medium in terms of the root length attained (Figure 4.16), in contrast the addition of IAA yielded a greater ratio of explants that actually rooted than that seen with IBA. For 1/2-strength B5 medium supplemented NAA could not be observed as the result of callus formation (Figure 4.19).

Table 4.4 Effect of auxins concentrations on root induction in *G. glabra* in 1/2-strength B5 medium

Auxins concentrations (mgL^{-1})			Rooting%	Length of roots (cm)
IAA	IBA	NAA		
0	0	0	50	2.26 ± 0.12^{A}
1.0	0	0	50	1.82 ± 0.43^{A}
2.0	0	0	60	1.20 ± 0.28^{A}
3.0	0	0	50	1.56 ± 0.26^{A}
4.0	0	0	40	1.12 ± 0.30^{A}
5.0	0	0	70	1.43 ± 0.22^{A}
0	0	0	50	2.71 ± 0.43^{A}
0	1.0	0	30	1.78 ± 0.58^{A}
0	2.0	0	30	0.90 ± 0.38^{A}
0	3.0	0	40	1.85 ± 0.36^{A}
0	4.0	0	40	1.80 ± 0.36^{A}
0	5.0	0	60	1.90 ± 0.34^{A}
0	0	0	-	-
0	0	1.0	-	-
0	0	2.0	-	-
0	0	3.0	-	-
0	0	4.0	-	-
0	0	5.0	-	-

Data represent mean value of 20 culture± S.E. Within a column, values with different superscript letters are significantly different ($P < 0.05$) using Scheffe's multiple range test.

Table 4.5 Effect of auxins and its concentrations on percentage of root induction of *G. glabra* on 1/2-strength B5 medium after culturing for 6 weeks

Concentration (mgL^{-1})	Root induction (%)		
	IAA	IBA	NAA
0	50	50	-
1	50	30	-
2	60	30	-
3	50	40	-
4	40	40	-
5	70	60	-

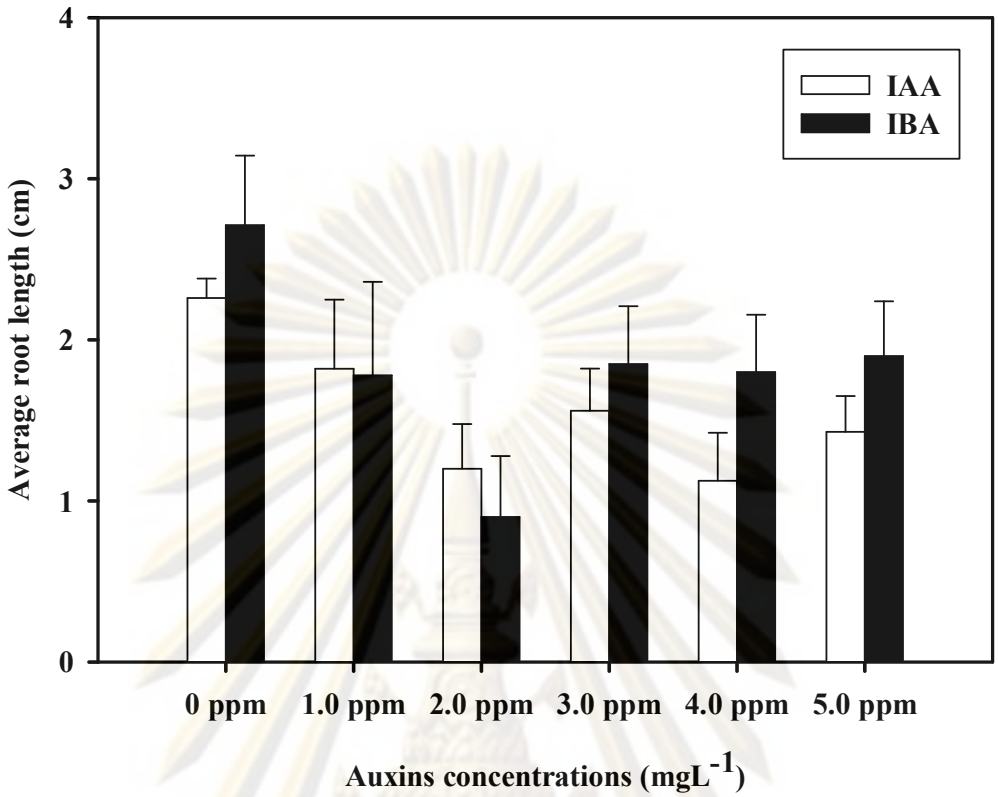

Figure 4.16 Effect of auxins and its concentrations on average root length of *G. glabra* after cultured on 1/2- strength B5 medium for 6 weeks. Each bar represents the mean ±S.E. of 20 replicates.

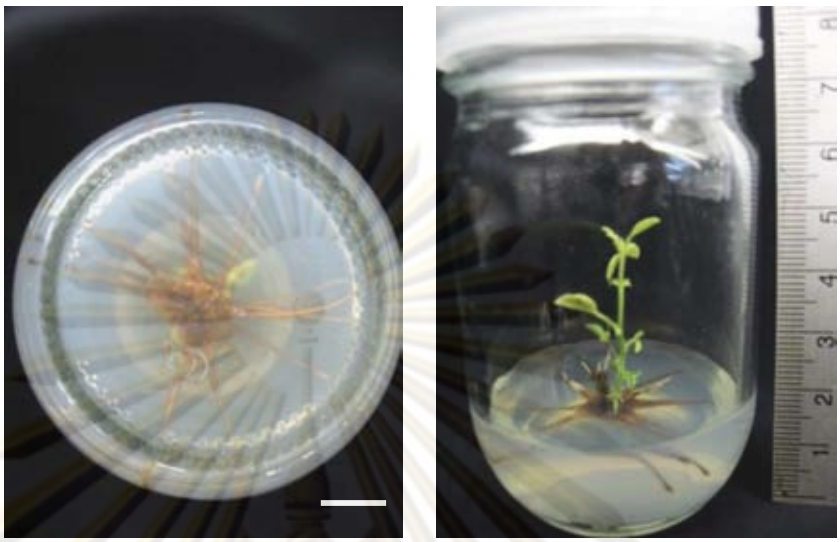

Figure 4.17 Rooting on 1/2 strength B5+ 5.0 mgL^{-1} IAA of *G. glabra* after cultured for 6 weeks.

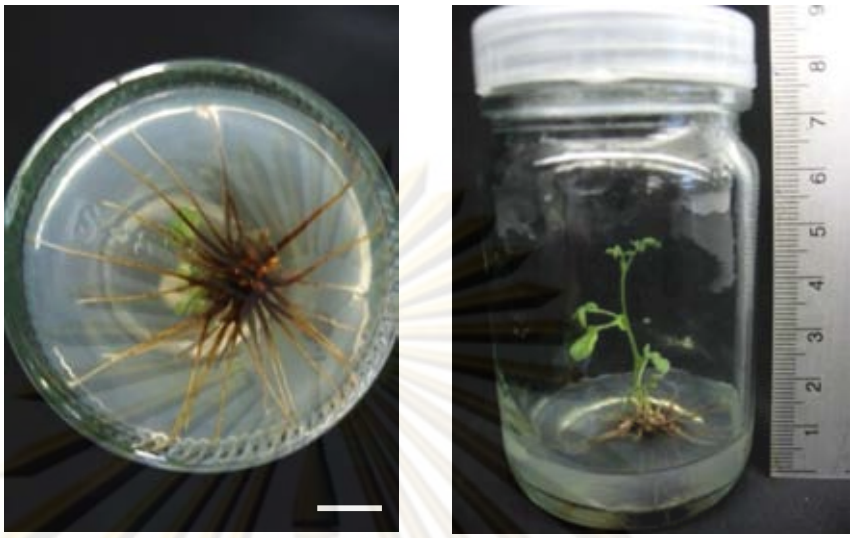

Figure 4.18 Rooting on 1/2 strength B5+ 5.0 mgL^{-1} IBA of *G. glabra* after cultured for 6 weeks.

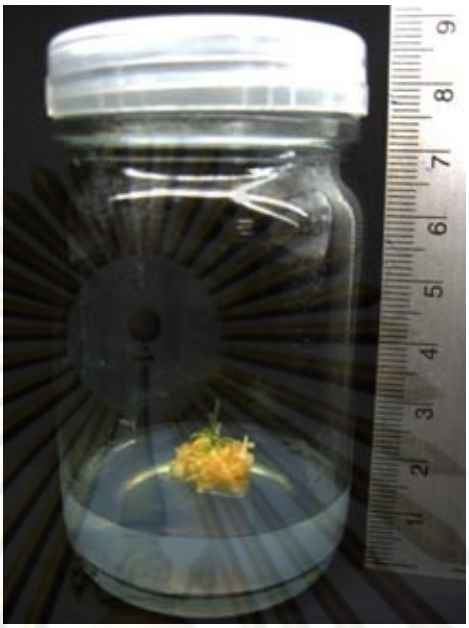

Figure 4.19 Callus derived from *G. glabra* shoot explants on 1/2 strength B5+2.0 mgL^{-1} NAA after cultured for 6 weeks.

4.1.5 Hardening of plantlets

The *in vitro* raised plantlets were subsequently transferred individually to plastic cups filled with sterile garden soil (SC1), vermiculite (SC2), garden soil mixed with vermiculite and ash husk at 1:1:1 (w/w) (SC3), ash husk (SC4) and garden soil mixed with ash husk at 1:1 (w/w) (SC5). These plantlets were kept at room temperature in natural light for about a week and then transferred to a greenhouse, where the surviving plantlets resumed normal growth and developed healthy leaves after two weeks. In the case of the SC1 media, some 95% of the plantlets survived (Table 4.6).

Table 4.6 The survival percentage of *G. glabra* affecting by different culture substrates (SC1, sterile garden soil; SC2, vermiculite; SC3, garden soil mixed with vermiculite and ash husk (1:1:1); SC4, ash husk; SC5, garden soil mixed with ash husk (1:1) observed after 3 weeks.

Culture substrates	% Survival
SC1	95
SC2	95
SC3	0
SC4	0
SC5	0

Figure 4.20 Plantlet which well developed roots of *G. glabra* after transfered to plastic bag containing various culture substrates; sterile garden soil (SC1), vermiculite (SC2), garden soil mixed with vermiculite and ash husk (1:1:1) (SC3), ash husk (SC4) and garden soil mixed with ash husk (SC5).

4.2 Quantitative analysis of glycyrrhizin in *G. glabra*

4.2.1 Effect of plant growth regulators on glycyrrhizin production

The effect of IAA or IBA on glycyrrhizin production of shoot explants cultured on 1/2-strength B5 medium was shown in Figure 4.21. The results showed that IAA and IBA gave higher stimulation of biosynthesis of glycyrrhizin production than control in every growth stages. It can be concluded that the secondary metabolic system of the plant is stimulated by the chemical stress to accelerate the production of glycyrrhizin. In addition, IAA also gave higher certain of glycyrrhizin than IBA at all the growth stages especially at the highest value on the eighth weeks.

4.2.2 Glycyrrhizin accumulation of culture during growth stages

Shoot cultures from 6 weeks old were cut and transferred to half-strength B5 medium. Each medium was supplemented with constant concentration at 5.0 mgL^{-1} of two auxins (IAA and IBA). Roots were harvested and analyzed at different time intervals. Three replicates of samples were maintained for each treatment conditions. The contents of glycyrrhizin were analyzed by HPLC. The time course of the effect of plant growth regulators on glycyrrhizin accumulation in 12 weeks old cultures is shown in Figure 4.21. After 8 weeks, the maximum content of glycyrrhizin in culture using 1/2- strength B5 medium supplemented with 5 mgL^{-1} IAA reached significantly to 27.57±0.66 $\mu g\ g^{-1}$ dry wt as compared with that of the non-supplemented control (0.57±0.29 $\mu g\ g^{-1}$ dry wt).

4.2.3 Variability in the content of glycyrrhizin between *in vitro* licorice root and natural licorice root

Two different source of natural root were purchased from local Chinese herbal stores in Bangkok while *in vitro* root derived from our own lab. Licorice root was extracted for analysis of glycyrrhizin detected by HPLC. According to the result of HPLC analysis described in Figure 4.22, 4.23 and 4.24, the chromatograms which two natural root extract were appeared any impurities in front of glycyrrhizin more than *in*

vitro root extract. But for the glycyrrhizin of those detected by HPLC were appeared similarly in retention time.

Table 4.7, illustrated the glycyrrhizin content ($\mu g g^{-1}$ dry wt) in licorice sample of *in vitro* root cultured on 1/2-strength B5 medium supplemented with 5 mgL^{-1} IAA after 8 weeks, natural root A and natural root B. It was clearly shown that natural root A and B gave the higher glycyrrhizin content more than *in vitro* root.

Table 4.7 Glycyrrhizin content ($\mu g g^{-1}$ dry wt) in licorice sample; *in vitro* root of licorice cultured on 1/2 B5+ 5 mgL^{-1}IAA after 8 weeks(A); natural root A(B); natural root B(C).

Licorice sample	Glycyrrhizin content ($\mu g g^{-1}$ dry wt)
A	27.57 ± 0.66
B	140.39 ± 1.44
C	393.16 ± 4.83

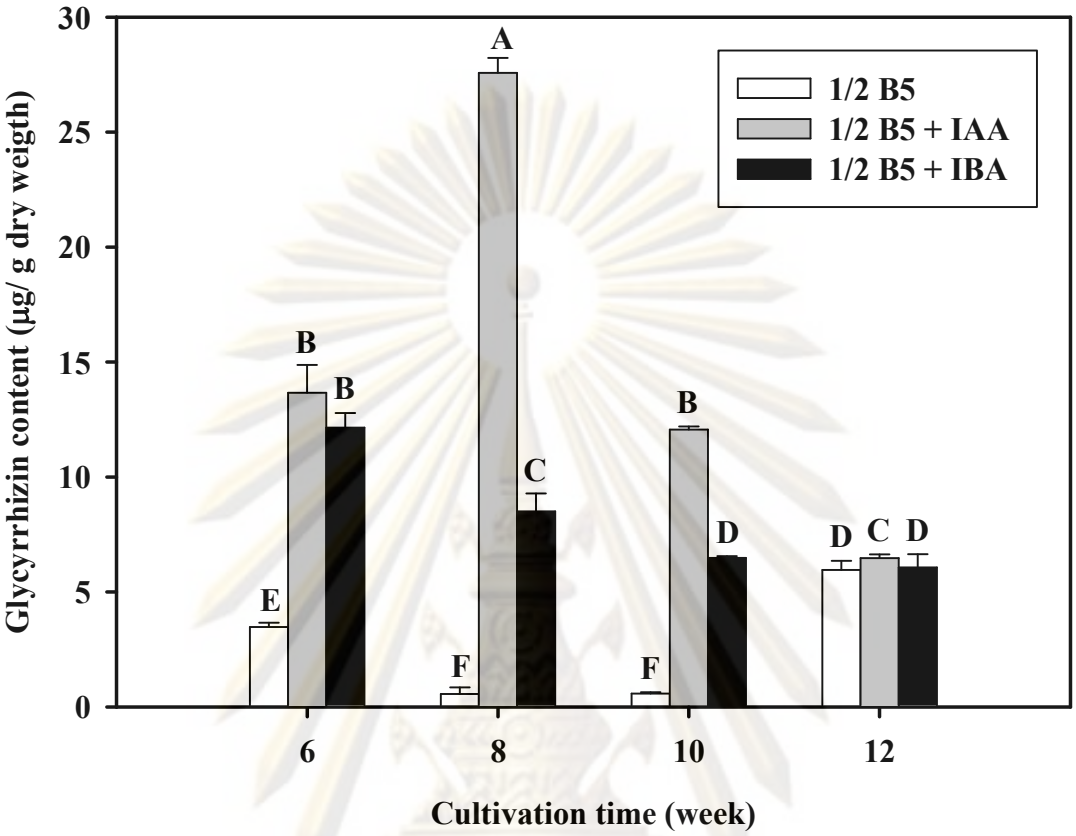

Figure 4.21 Glycyrrhizin accumulation in culture of *G. glabra* using 1/2-strength B5 supplemented with 5 mgL^{-1}IAA or IBA harvested at different cultivation time. Each bar represents the mean ±S.E. of 20 replicates. Statistical significance among the treatments at $P<0.05$ indicated by A, B, C, D, E, F was determined by Scheffe's multiple range test.

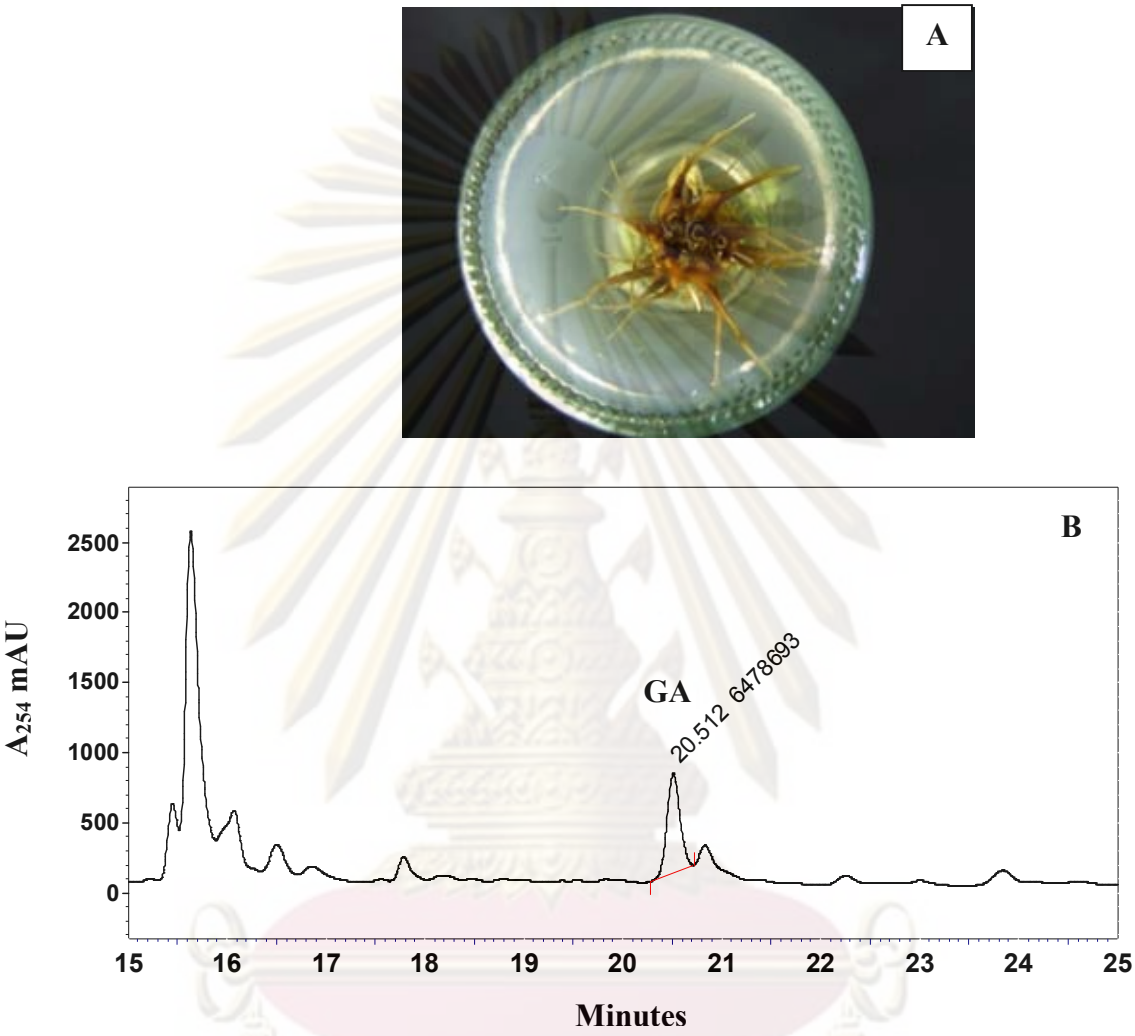

Figure 4.22 *In vitro* roots of *G. glabra*

(A) Rooting derived from the cultured of licorice on 1/2-strength B5 medium supplemented with 5 mgL^{-1}IAA after 8 weeks which use for glycyrrhizin detection.

(B) HPLC profile of the crude sample extracted from *In vitro* root of licorice for the glycyrrhizin (GA) detected at 254 nm.

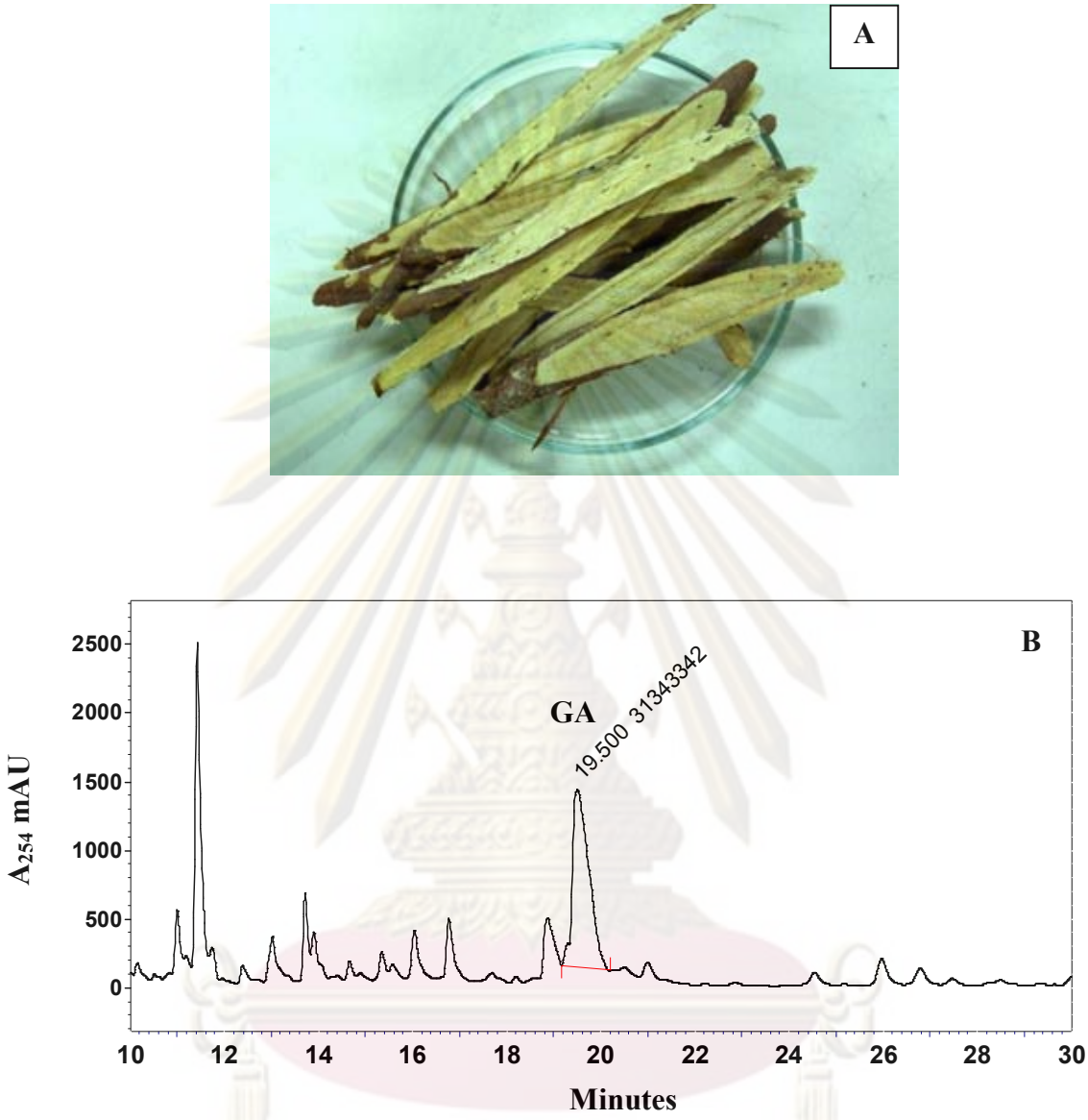

Figure 4.23 Natural root A (A); HPLC profile of the crude sample extracted from natural root A of licorice for the analysis of glycyrrhizin detected at 254 nm (B).

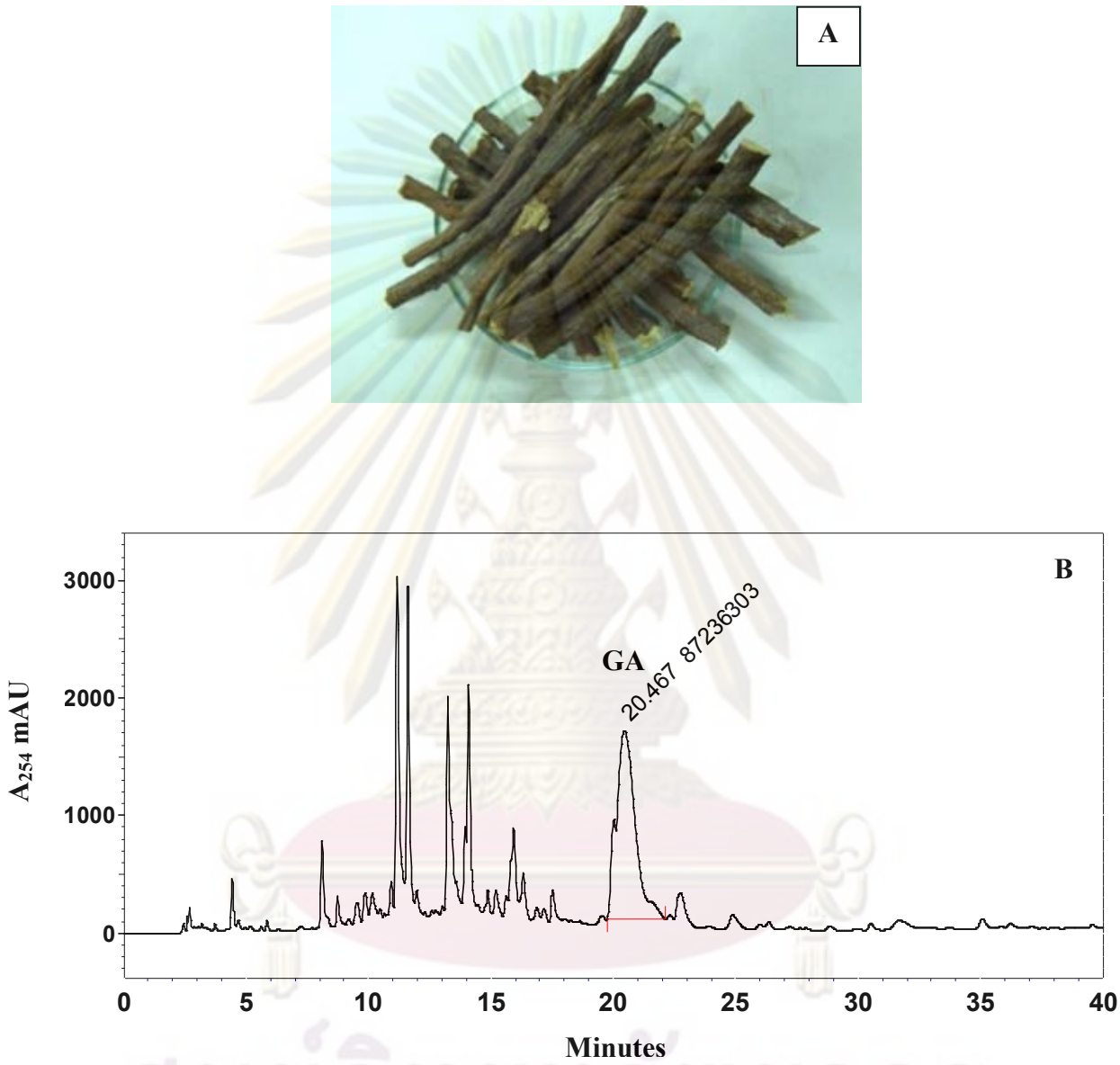

Figure 4.24 Natural root B (A); HPLC profile of the crude sample extracted from natural root B of licorice for the analysis of glycyrrhizin detected at 254 nm (B).

CHAPTER V

DISCUSSION

5.1 Tissue culture of *G. glabra*

5.1.1 Effect of medium formulations and concentration on shoots growth and development

As for plant development, tissue culture is a good means of starting for shoot tips explants. Under total artificial environment, different species of cultured plant may require different nutrient. Even the tissue from the different parts of the same plant may differ in nutritional requirement for satisfactory growth (Murashige and Skoog 1962). That mean the composition of basal culture medium was an important factor for the successful establishment of tissue culture in which each tissue required different formulation (Dixon 1990). No doubt, the MS is the most common medium formulation and proved to be satisfactory in tissue culture of many plant species for micropropagation, several others are used regularly. Tissue culture of *G. glabra* was chosen in this study using different media (MS, B5 and WPM) for shoots growth and development under different concentration of salt base (1-, 1/2-, 1/4-strength). After six weeks of culture, morphogenic changes in the cultured explants were clearly observed. The favorable effect of a low concentration of MS macronutrients, also noted in this study (data not shown), has been discussed by many authors, as has the increased rate of multiplication when using MS salts at 3/4-, 1/2- and 1/4- strength (Hyndman *et al*., 1982). In the present study, however, media formulation additionally was observed to display a clear effect upon the morphogenic responses that were observed. In overall, 1/2-strength B5 salts were the most suitable media for the *in vitro* tissue culture growth and morphogenesis of *G. glabra* plants (Table 4.1). In accordance with the Gamborg's B5 medium, it was devised for the culture of legume (leguminosae) and contains a much greater proportion of nitrate compared to the ammonium ion. This may indicate that B5 medium had significant effects on shoot growth and development at low concentration of salt base.

Furthermore, 1/4-strength MS medium with well-figured concentration of ammonia gave the shoot healthy shoot and more uniform in stem diameter and even more leaves and stronger branches. The reason for this is the concentration of ammonium used in plant nutrient media. At low concentration of ammonium salt in media could stimulate growth and development as for using WPM, poor growth and development on the external morphology in culture explants was obtained. However, despite the general beneficial effect of the reduced macronutrient level of media on *G. glabra* plant growth and differentiation (Table 4.1), the maximum number of shoots per explant (1.5) was found when explants were grown on full-strength MS salts, but still this was only marginally higher than that on half-strength MS. Therefore, the effect of cytokinins at different concentrations upon shoot multiplication rates was evaluated in explants cultured under full-strength MS salts.

5.1.2 Effect of sucrose concentration on shoots growth and development

Several authors have been reported the influence of the carbon source on the *in vitro* morphogenesis of different plant species. Among the many available carbon sources, sucrose has been the major role (Petersen *et al.,* 1999; Fuentes *et al.,* 2000). Sugar and basal medium are necessary components of plant tissue culture medium. Sugar concentration and strength of basal medium was studied on conversion of somatic embryos in *Asparagus officinalis* L. but, there is no report about the effects of sugar and basal medium on growth and development of shoot explants in *G. glabra.*

Table 4.2 shows the effects of concentration of sugar on shoot length, stem diameter, leaf width and number of branches. The results of this study show that the growth of shoot length and number of branches from shoot explants cultured under 1/4-strength MS medium gave a higher value when compared with 1/2-strength B5 medium at 10 and 30 gL^{-1} sucrose concentration. It was suggested that lower concentrations of sugar under 1/4-strength MS medium promoted the growth of shoot length and number of branches and inhibited the growth of stem diameter and leaf width. In contrast, the results of stem diameter and leaf width from the explants cultured under 1/2-strength B5 medium gave a higher value when compared with 1/4-strength MS medium at sucrose concentration of ranging from 30 - 60 gL^{-1}. It was also suggested that higher concentrations of sugar of 1/2-strength B5 medium promoted the growth of stem diameter and leaf width so as to inhibit the growth of

shoot length and number of branches. As a result, 30 gL^{-1} of sucrose concentration should get fine morphological shoot structure.

5.1.3 Effect of cytokinin on shoot multiplication

Cytokinins were used mostly to induce shoot bud induction, development and multiplication. It stimulated cell division and both formation and growth of axillary and adventitious shoots in plant tissue culture. Both naturally occurring and synthetic Cytokinins are used in plant culture media. In this study, synthetic cytokinins including benzyladenine (BA), 6- furfurylaminopurine (Kinetin) and thidiazuron (TDZ) are used to inducing shoot multiplication due to the lowering cost.

Cytokinin in full-strength MS medium was used for shoot tip as a explants source. Certainly, the *in vitro* multiplication of *G. glabra* using different explants sources on modified MS media has been reported previously for seedling explants (Shah and Dalai, 1980) and cultures (Syrtanova and Mukhitdinova, 1984), as well as a high frequency regeneration of *G. glabra* from nodal explants. Moreover, whilst successful shoot tip explant cultivation in simple media has been reported before (Thengane *et al*., 1998) study, however, shoot explants were successfully cultured for six weeks on MS medium, and that supplemented with 0.5 mgL^{-1} BA was found to be the most effective media resulting highest number of shoots per explants but also the highest shoot length. This is in agreement with previous reports that a low cytokinin concentration without any exogenously added Auxins plays an important role in shoot induction (Kohjyouma *et al.*, 1995; Thengane *et al.*, 1998). Hence, media supplemented with the cytokinin BA at 0.5 mgL^{-1} was selected as the appropriate condition for shoot proliferation assays in the following experiment.

5.1.4 Effect of auxin types on rooting ability

Shoot tip also used as an explants source cultured on 1/2-strength B5 medium supplemented with Auxin which are known to affect a root formation. Natural and synthetic Auxins are commonly used in tissue culture. Natural occurring Auxins include Indole-3-acetic acid (IAA) and Indole-3-butyric acid (IBA). At present, IBA is considered to be synthetic, but has been found to occur naturally in many plants including olive and tobacco (Epstein *et al.,* 1989). Synthetic Auxins which are often

used in plant tissue culture are 1-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D). Since 2,4-D has been widely used in plant tissue, for its ability to inhibit the production of secondary metabolites in a large number of cases (Rajendran *et al*., 1992). Therefore, during experiment, Auxins have been used including IAA, IBA and NAA as to induce adventitious root formation.

Optimum auxin concentration is determined based on percentage of root induction and root length. Table 4.5, illustrated the effect of auxins on percentage of root induction from shoot explants which were cultured on 1/2-strength B5 medium for 6 weeks. In this study, it was found that a high percentage of root induction was obtained on the medium with 5.0 mgL^{-1} of either IAA (70%) or IBA (60%). Figure 4.16 shows the Effect of auxins on root length of *G. glabra* after cultured on 1/2-strength B5 medium for 6 weeks. The result shown that 3.0-5.0 mgL^{-1} IBA gave a high value in root length and obtained a healthy root. However, neither a significant difference among the kinds of auxin nor a significant interaction was shown. This result differ from the results of Thengane *et al.* (1998). They found that the combination of auxins (0.5 mgL^{-1}IAA + 1.0 mgL^{-1}IBA) yielded the maximum value about 6.25±1.78 mm in root length.

Another case found was those medium containing no auxins or control on root length. It gave the highest value. This occurrence may be caused by whether the existing of auxins itself in those tissues is sufficient for root induction. But for the percentage of root induction shown to decrease rooting.

In case of explants grown on medium containing NAA, the callusing was presence at every level of NAA. The data could not be observed. The use of NAA is more limited than other Auxins. Mostly, it is used mostly in callus induction and at much lower concentrations than other auxins.

5.1.5 Hardening of plantlets

Normally, the greenhouse and field have lower relative humidity, higher light level and septic environment that cause stress to micropropagated plants as compared to *in vitro* conditions, thus plantlets grown *in vitro* require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transfer to soil. Acclimatization is an importance step in micropropagation. During *in vitro* culture, plant develop under controlled conditions, including enclosed

environments, no gas exchange, high moisture in the air, low light intensity, and the use of sugars from the medium as a carbon source and energy (Preece and Sutter, 1991). Therefore, the transplanting of *in vitro*-grown plantlets and complete establishment in the greenhouse can be complex for some species (Van Huylenbroeck and Debergh, 1996). The selection of a suitable substrate can be decisive for acclimatization process.

Table 4.6 illustrated, the survival rate of *G. glabra* affecting by different substrates culture (SC1, sterile garden soil; SC2, vermiculite; SC3, garden soil mixed with vermiculite and ash husk (1:1:1); SC4, ash husk; SC5, garden soil mixed with ash husk (1:1) observed after 3 weeks. The substrates used for acclimatization process revealed that SC1 and SC2 have survival percentage 95% either. Soil provided good aggregation to root water retention, whereas vermiculite had excellent water storage. On the other hand, plant survival rate was zero for SC3, SC4 and SC5. This result indicated that those substrates mixed with ash husk were not suitable for acclimatization process. Although ash husk had a great drainage, it had a pH greater than 7 which negatively affected plant development and the use of ash husk mixture should not be recommended for the acclimatization of this plant. Therefore, SC1 was selected as a proper substrate for acclimatization process because the cost of these materials was reasonably cheap.

5.2 Quantitative analysis of glycyrrhizin in *G. glabra*

5.2.1 Effect of plant growth regulators on glycyrrhizin production

Production of secondary metabolites can be enhanced or reduced by changing the composition of the nutrient medium, carbon supply, plant growth regulators and the stage of growth of the culture. Growth regulator concentration is often a crucial factor in secondary product accumulation (DiCosmo and Towers, 1984; Deus and Zenk, 1982). The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio alters dramatically both the growth and the product formation in cultured plant cells. (Mantell and Smith, 1984)

According to the previous study, a successful result was obtained for root induction of *G. glabra* Linn. from shoot explants cultured under 1/2-strength B5

medium supplemented with 5.0 mgL^{-1} IAA or IBA using an appropriate level to achieve high percentage of root induction and root length.

Recently, previous work has reported the failure to detect *in vitro* production of glycyrrhizin in *G. glabra* L. (Wu *et al*., 1974; Hayashi *et al*., 1988) despite they were found to contain several triterpenes. Indeed, although Ko *et al*. (1989) reported the production of glycyrrhizin in transformed hairy root cultures of *G. ulalensis*, they found no production in transformed roots of *G. glabra*. Wongwicha *et al*. (2008) recently reported that *G. ulalensis* calli induced using MS + NAA and BA or TDZ alone produced glycyrrhizin, as discussed above. However, Shams-Ardakani *et al*. (2007) similarly reported the formation of glycyrrhizin from *G. glabra* var. *glandulifera* calli tissue induced using MS + 2, 4-D or MS+NAA + 2,4-D + kinetin.

Figure 4.21 showed that the variation in the glycyrrhizin content was clearly attributed to the type and concentration of plant growth regulator, which dramatically altered both the growth and their glycyrrhizin formation in plant tissue culture. The result also showed that IAA or IBA gave higher stimulation of biosynthesis of glycyrrhizin production than 'control' in every growth stages. In addition, the cultures grown on medium containing IAA gave higher quantity of glycyrrhizin than IBA at all growing stages especially at the highest value on the eight weeks. These means IAA would enhance the production of glycyrrhizin. This is potential dependence upon the auxin/cytokinin ratio reported here in accord with the broad trend also observed for tissue culture of *G. ulalensis* explants (Wongwicha *et al*., 2008).

5.2.2 Glycyrrhizin accumulation of culture during growth stages

There was no report before for the production of glycyrrhizin which was stimulated by plant growth regulator using as chemical stress. The results shown in Figure 4.21 indicated the time course for glycyrrhizin accumulation in the cultured explants varied significantly, presumably reflecting large differences in glycyrrhizin formation during tissue development and differentiation. The maximum content of glycyrrhizin in culture after 8 weeks under 1/2- strength B5 medium supplemented with 5 mgL^{-1} IAA reached to 27.57±0.66 $\mu g\ g^{-1}$ dry wt as compared with that of the non-supplemented control (0.57±0.29 $\mu g\ g^{-1}$ dry wt). In term of 5 mgL^{-1} IBA, it was shown that the highest production of glycyrrhizin in cultures on weeks 6 reached to 12.14±0.64 $\mu g\ g^{-1}$ dry wt. That means that the biosynthesis of glycyrrhizin might be

induced by IAA more than IBA. The production of glycyrrhizin stimulated in response to 5 mgL^{-1} IAA increased significantly with time up to week 8 and then decreased from 8 to 12 weeks. The most notable conclusions were that secondary accumulation, in general, was enhanced by plant growth regulators and that maximum accumulation took place at the late time of growth. The depletion of secondary accumulation may be caused by the decomposing of plant growth regulators when cultures were carried out in light over time. Another possibility is that the secondary metabolites may not be stable enough, degraded ultimately to inactive culture and stored carbon recycled back for balancing between the activities of primary and secondary metabolism, which would be largely affected by growth, tissue differentiation and development of plant body.

5.2.3 Variability in the content of glycyrrhizin between *in vitro* licorice root and natural licorice root

Plant tissue culture usually produce low amounts of secondary metabolites compared with the intact plant. This is due to the biochemical defence mechanism against pathogens and predators (Bennet and Wallsgrove, 1994). These factors, which dependent of location, and accumulation of secondary products in the intact plant, and since elicitation of secondary pathway by pathogen will lead to the localized production of phytoalexin or secondary metabolites. In addition, the long cultivation periods also used to produce high-yielding secondary metabolites.

Nowadays, the plant cell culture technology is sufficiently advanced to generate for the production of secondary metabolites (Fontanel and Tabata, 1987; Dicosmo *et al.* 1989; Dixon and Lamb, 1990). Several products are accumulated in cultured cells at a higher level than found in intact plants. For example, berberine by *Coptis japonica* (Matsubara *et al.* 1989), ginsenosides by *Panex ginseng* (Ushiyama, 1991), rosmarinic acid by *Coleus blumei* (Ulbrich *et al.* 1985), shikonin by *Lithospermum erythrorhizon* (Takahashi and Fujita, 1991), diosgenin by *Dioscorea* (Matsumoto *et al.* 1980), ubiquinone-10 by *Nicotiana tabacum* (Fontanel and Tabata, 1987) were accumulated in cultured cells more than in the intact plant.

As shown in Table 4.7, the amount of glycyrrhizin content of natural root A and natural root B gave higher rate than those *in vitro* roots. However, this research succeeded in using plant tissue culture technologies to produce glycyrrhizin better

than ‘control’ in the short period. In order to obtain high yielding as for commercial purposes, the protocol was established in this study which would enable for future works on the stimulation of biosynthetic activities of cultured cell using various methods include optimizing nutrient components, adding precursors and regulators, inducing hairy root culture and employing elicitors.

CHAPTER VI

CONCLUSION

The present work could be concluded as follows:

1. The effective media for shoot growth and development of *G. glabra* was 1/2-strength B5 medium.

2. Addition of 30 gL^{-1} sucrose on 1/2-strength B5 medium or 1/4-strength MS medium gave the high efficiency of shoot growth that promoted shoot length, stem diameter, leaf width and number of branches.

3. The optimum conditions for shoot multiplication on number of shoots and shoot length of *G. glabra* from shoot explants can be accomplished on full strength MS medium supplemented with 0.5 mgL^{-1} BA.

4. The optimum conditions for root induction on percentage and root length of *G.* glabra from shoot explants were established on 1/2-strength B5 medium supplemented with 5.0 mgL^{-1} IBA or IAA.

5. Sterile garden soil was selected as a proper substrate for acclimatization process.

6. The presence of plant growth regulators of IAA or IBA 5.0 mgL^{-1} gave higher stimulation of biosynthesis of glycyrrhizin production than control in every growth stages.

7. The maximum content of glycyrrhizin in culture after 8 weeks was 1/2- strength B5 medium supplemented with 5 mgL^{-1} IAA. In addition, IAA also gave higher quantity of glycyrrhizin than IBA at all the growth stages.

8. Natural roots yield higher glycyrrhizin content than *in vitro* roots.

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APPENDICES

APPENDIX A

Composition of plant tissue culture media

1.1 Murashige and Skoog medium (1962)

Macronutrients	**mgL^{-1}**
KNO_3	1900
NH_4NO_3	1650
$MgSO_4..7H_2O$	370
$CaCl_2.2H_2O$	440
KH_2PO_4	170

Micronutrients	**mgL^{-1}**
$MnSO_4.4H_2O$	22.3
KI	0.83
H_3BO_3	6.2
$ZnSO_47H_2O$	8.6
$CuSO_4.5H_2O$	0.025
$Na_3MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025

Iron	**mgL^{-1}**
$FeSO_4.7H_2O$	27.8
Na_2EDTA	37.3

Organic compounds	**mgL^{-1}**
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1
myo-Inositol	100
Glysine	2.0

Sucrose **30 gL^{-1}**

pH **5.7**

1.2 Gamborg medium (1968)

Macronutrients	**mgL^{-1}**
KNO_3	2500
$(NH_4)_2SO_4$	134
$MgSO_4.7H_2O$	250
$CaCl_2.2H_2O$	150
$NaH_2PO_4.H_2O$	150

Micronutrients	**mgL^{-1}**
$MnSO_4.H_2O$	10
KI	0.75
H_3BO_3	3.0
$ZnSO_47H_2O$	2.0
$CuSO_4.5H_2O$	0.025
$Na_3MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	0.025

Iron	**mgL^{-1}**
$Feso_4.7H_2O$	27.8
Na_2EDTA	37.3

Organic compounds	**mgL^{-1}**
Nicotinic acid	1.0
Pyridoxine-HCl	1.0
Thiamine-HCl	1.0
myo-Inositol	10

Sucrose 30 gL^{-1}

pH 5.7

1.3 Woody Plant Medium

Macronutrients	**mgL^{-1}**
NH_4NO_3	400
$MgSO_4..7H_2O$	370
$CaCl_2.2H_2O$	96
KH_2PO_4	170
$Ca(NO_3)_2.4H_2O$	556

Micronutrients	**mgL^{-1}**
$MnSO_4.H_2O$	29.4
H_3BO_3	6.2
$ZnSO_47H_2O$	8.6
$CuSO_4.5H_2O$	0.25
$Na_3MoO_4.2H_2O$	0.25

Iron	**mgL^{-1}**
$Feso_4.7H_2O$	27.8
Na_2EDTA	37.3

Organic compounds	**mgL^{-1}**
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1
myo-Inositol	100
Glycine	2.0

Sucrose 30 gL^{-1}

pH 5.7

APPENDIX B

Preparation of stock solution of media

2.1 Preparation of stock solution of MS media

Stock I

Macronutrients	mgL^{-1}	$10X(gL^{-1})$	20X(g/200ml)
KNO_3	1900	19	7.6
NH_4NO_3	1650	16.5	6.6
$(NH_4)_2SO_4$	-	-	-
$MgSO_4.7H_2O$	370	3.7	1.48
$CaCl_2.2H_2O$	440	4.4	1.76
KH_2PO_4	170	1.7	0.68
$NaH_2PO_4.H_2O$	-	-	-

Stock II

Micronutrients	mgL^{-1}	$100X(gL^{-1})$	100X(g/200ml)
$MnSO_4.H_2O$	-	-	-
$MnSO_44H_2O$	22.3	2.23	0.446
KI	0.83	0.083	0.0166
H_3BO_3	6.2	0.62	0.124
$ZnSO_47H_2O$	8.6	0.86	0.172
$CuSO_4.5H_2O$	0.025	0.0025	0.0005
$Na_3MoO_4.2H_2O$	0.25	0.025	0.005
$CoCl_2.6H_2O$	0.025	0.0025	0.0005

Stock III

Iron	mgL^{-1}	$100X(gL^{-1})$	100X(g/200ml)
$FeSO_4.7H_2O$	27.8	2.78	0.556
Na_2EDTA	37.3	3.73	0.746

Stock VI

Organic compounds	mgL^{-1}	$100X(gL^{-1})$	100X(g/200ml)
Nicotinic acid	0.5	0.05	0.01
Pyridoxine-HCl	0.5	0.05	0.01
Thiamine-HCl	0.1	0.01	0.002
myo-Inositol	100	10	2
Glycine	2.0	0.2	0.04

2.2 Prepatation of stock solution of B5 media

Stock I

Macronutrients	mgL^{-1}	$10X(gL^{-1})$	20X(g/200ml)
KNO_3	2500	25	10
NH_4NO_3	-	-	-
$(NH_4)_2SO_4$	134	1.34	0.536
$MgSO_4.7H_2O$	250	2.5	1
$CaCl_2.2H_2O$	150	1.5	0.6
KH_2PO_4	-	-	-
$NaH_2PO_4.H_2O$	150	1.5	0.6

Stock II

Micronutrients	mgL^{-1}	$100X(gL^{-1})$	100X(g/200ml)
$MnSO_4.H_2O$	10	1	0.2
$MnSO_44H_2O$	-	-	-
KI	0.75	0.075	0.015
H_3BO_3	3.0	0.3	0.06
$ZnSO_47H_2O$	2.0	0.2	0.04
$CuSO_4.5H_2O$	0.025	0.0025	0.0005
$Na_3MoO_4.2H_2O$	0.25	0.025	0.005
$CoCl_2.6H_2O$	0.025	0.0025	0.0005

Stock III

Iron	mgL^{-1}	$100X(gL^{-1})$	100X(g/200ml)
$FeSO_4.7H_2O$	27.8	2.78	0.556
Na_2EDTA	37.3	3.73	0.746

Stock VI

Organic compounds	mgL^{-1}	$100X(gL^{-1})$	100X(g/200ml)
Nicotinic acid	1.0	0.1	0.02
Pyridoxine-HCl	1.0	0.1	0.02
Thiamine-HCl	10.0	1	0.2
myo-Inositol	100	10	2
Glycine	-	-	-

2.3 Preparation of stock solution of WPM

Stock I

Macronutrients	mgL^{-1}	$10X(gL^{-1})$	20X(g/200ml)
KNO_3	-	-	-
NH_4NO_3	400	4	1.6 g/200ml
$Ca(NO_3)_2.4H_2O$	556	5.56	2.224
$MgSO_4.7H_2O$	370	3.7	1.48
$CaCl_2.2H_2O$	96	0.96	0.384
KH_2PO_4	170	1.7	0.68
$NaH_2PO_4.H_2O$	150	1.5	0.6

Stock II

Micronutrients	mgL^{-1}	$100X(gL^{-1})$	100X(g/200ml)
$MnSO_4.H_2O$	29.4	2.94	0.588
$MnSO_44H_2O$	-	-	-
KI	-	-	-
H_3BO_3	6.2	0.62	0.124
$ZnSO_47H_2O$	8.6	0.86	0.172
$CuSO_4.5H_2O$	0.25	0.025	0.005
$Na_3MoO_4.2H_2O$	0.25	0.025	0.005
$CoCl_2.6H_2O$	-	-	-

Stock III

Iron	mgL^{-1}	$100X(gL^{-1})$	100X(g/200ml)
$FeSO_4.7H_2O$	27.8	2.78	0.556
Na_2EDTA	37.3	3.73	0.746

Stock VI

Organic compounds	mgL^{-1}	$100X(gL^{-1})$	100X(g/200ml)
Nicotinic acid	0.5	0.05	0.01
Pyridoxine-HCl	0.5	0.05	0.01
Thiamine-HCl	0.1	0.01	0.002
myo-Inositol	100	10	2
Glycine	2.0	0.2	0.04

APPENDIX C

Preparation of stock solution (1000 ppm or 1000 mgL^{-1}) of cytokinins and auxins.

Cytokinins	**mg/200ml.**
BA	40
Kinetin	40
TDZ	40
Auxins	
IAA	40
IBA	40
NAA	40

APPENDIX D

Medium preparation

Mineral salts and vitamin mixtures may be prepared as stock solutions ranging from 10-1000 times the final concentrations. Stock solutions may be prepared as four solutions that contain all macronutrients, micronutrients, iron and organic compounds. The culture media contained 3.0% (w/v) sucrose as a carbon source. The pH of medium was adjusted to 5.7 using 1N NaOH and 1N HCl before gelling with agar (0.7% w/v) and 15 ml of medium were dispensed in 4 oz. bottles and autoclaved at 1.5 atm of pressure, 121°C for 15 min.

APPENDIX E

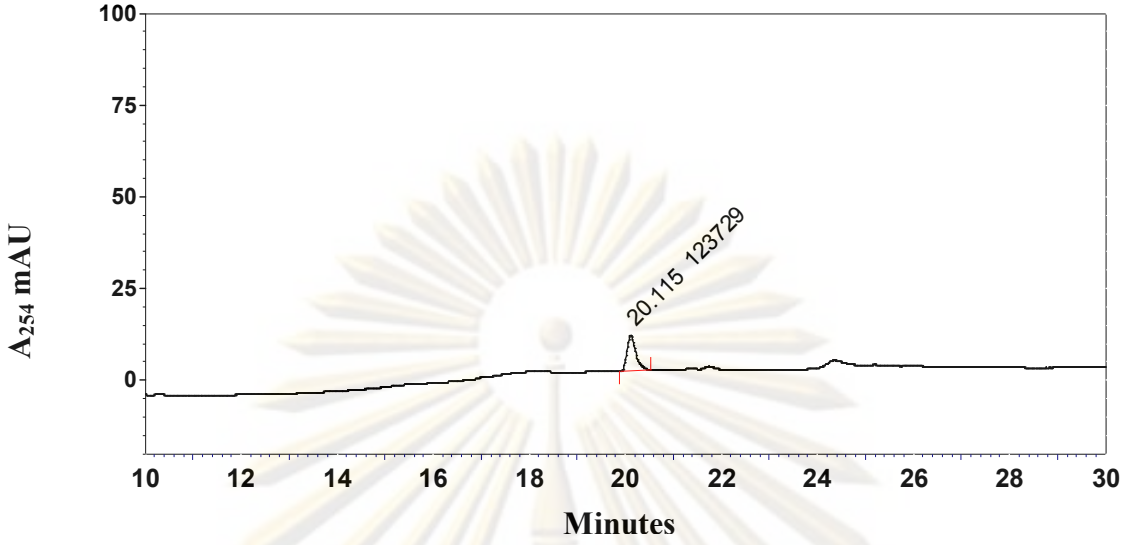

Figure E1 HPLC chromatograms of standard glycyrrhizin 2.5 ppm

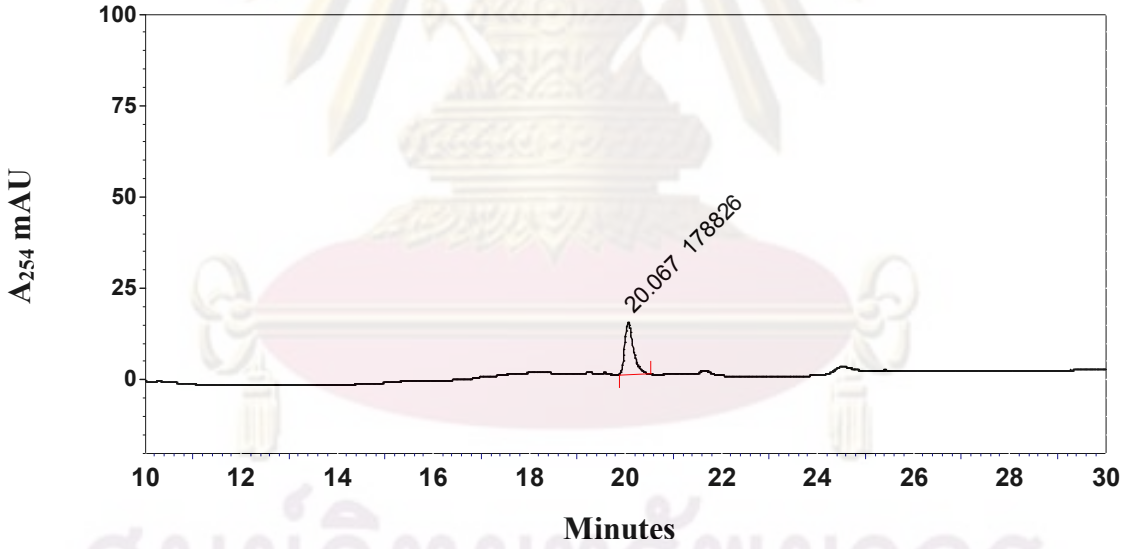

Figure E2 HPLC chromatograms of standard glycyrrhizin 5 ppm.

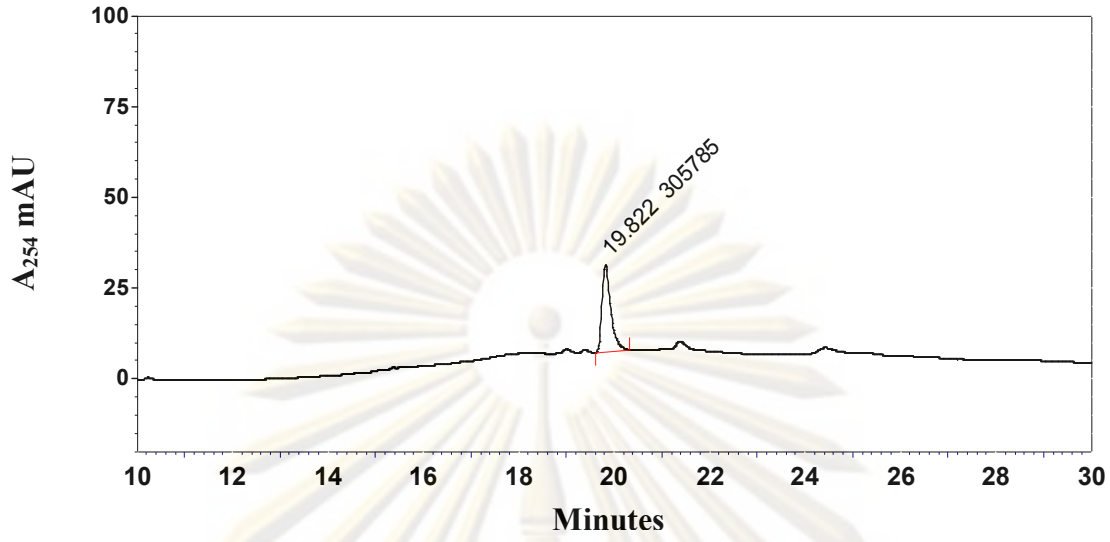

Figure E3 HPLC chromatograms of standard glycyrrhizin 12.5 ppm.

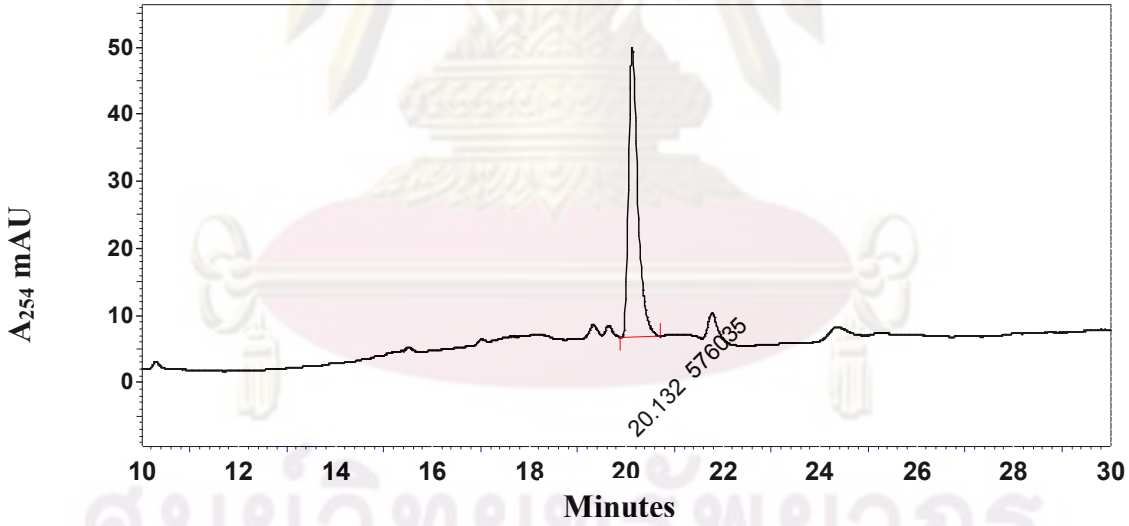

Figure E4 HPLC chromatograms of standard glycyrrhizin 25 ppm.

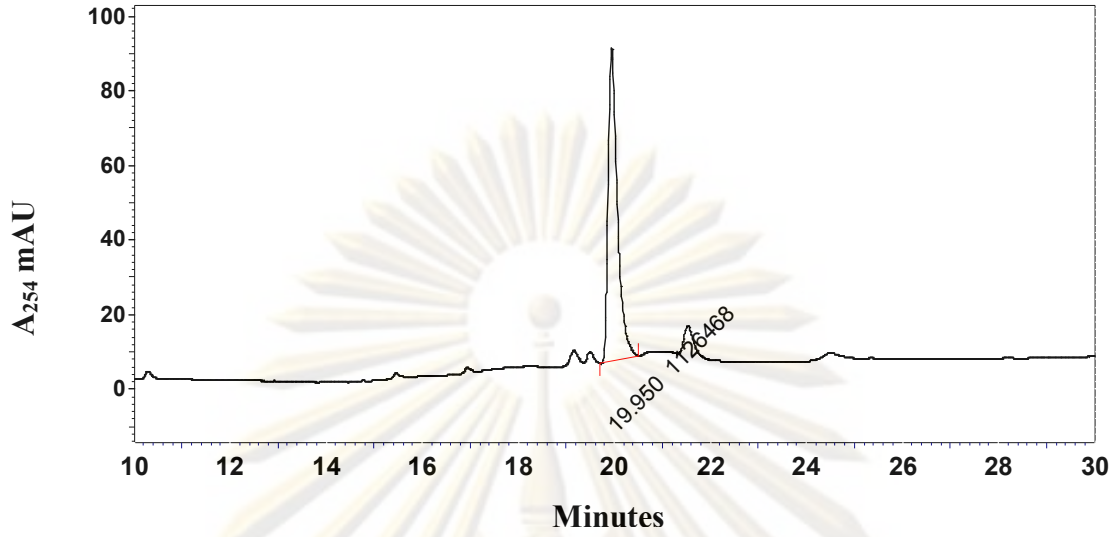

Figure E5 HPLC chromatograms of standard glycyrrhizin 50 ppm.

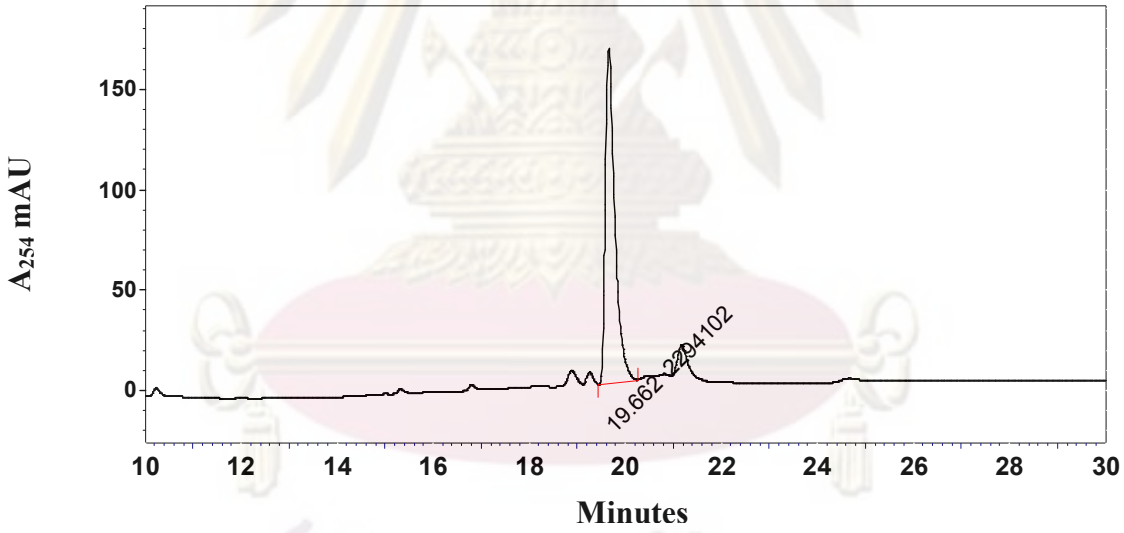

Figure E6 HPLC chromatograms of standard glycyrrhizin 100 ppm.

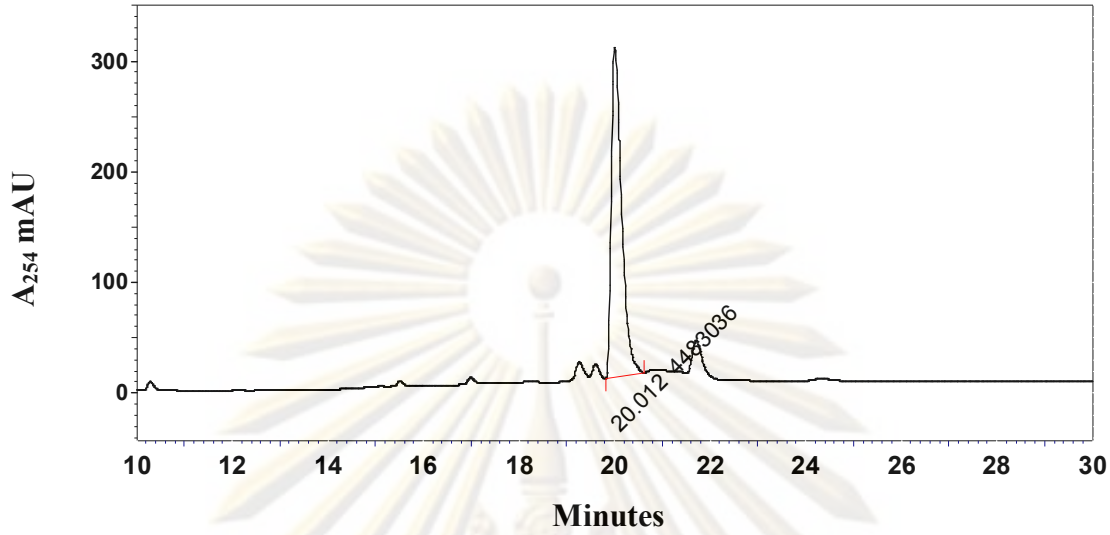

Figure E7 HPLC chromatograms of standard glycyrrhizin 200 ppm.

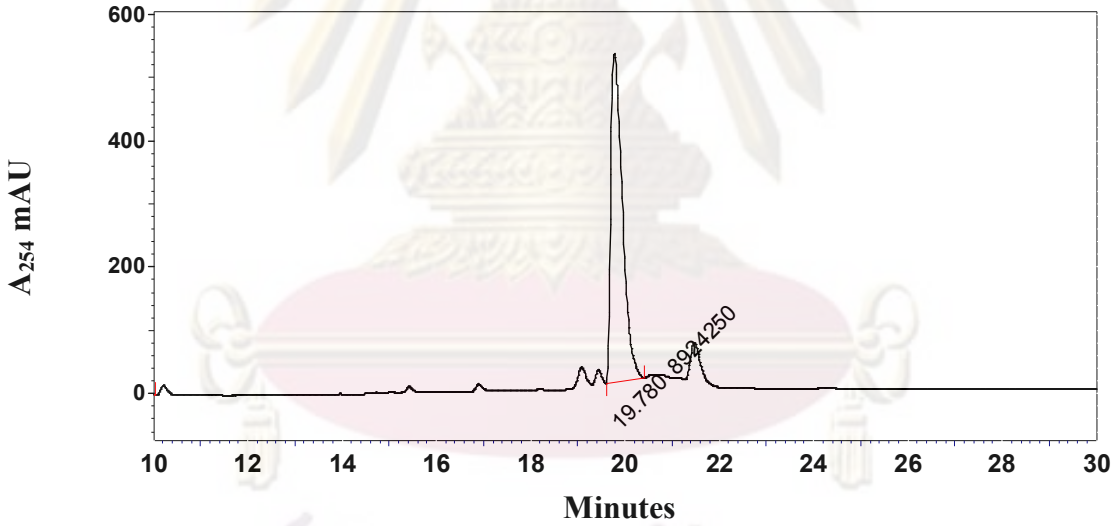

Figure E8 HPLC chromatograms of standard glycyrrhizin 400 ppm.

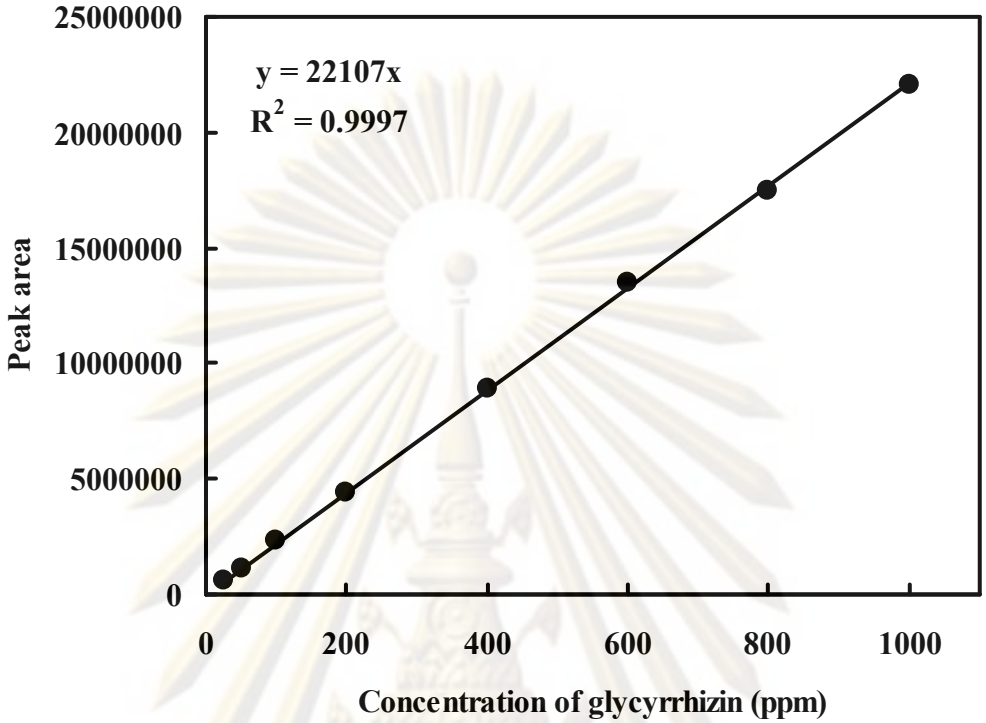

Figure E9 Standard calibration curve of glycyrrhizin.

APPENDIX F

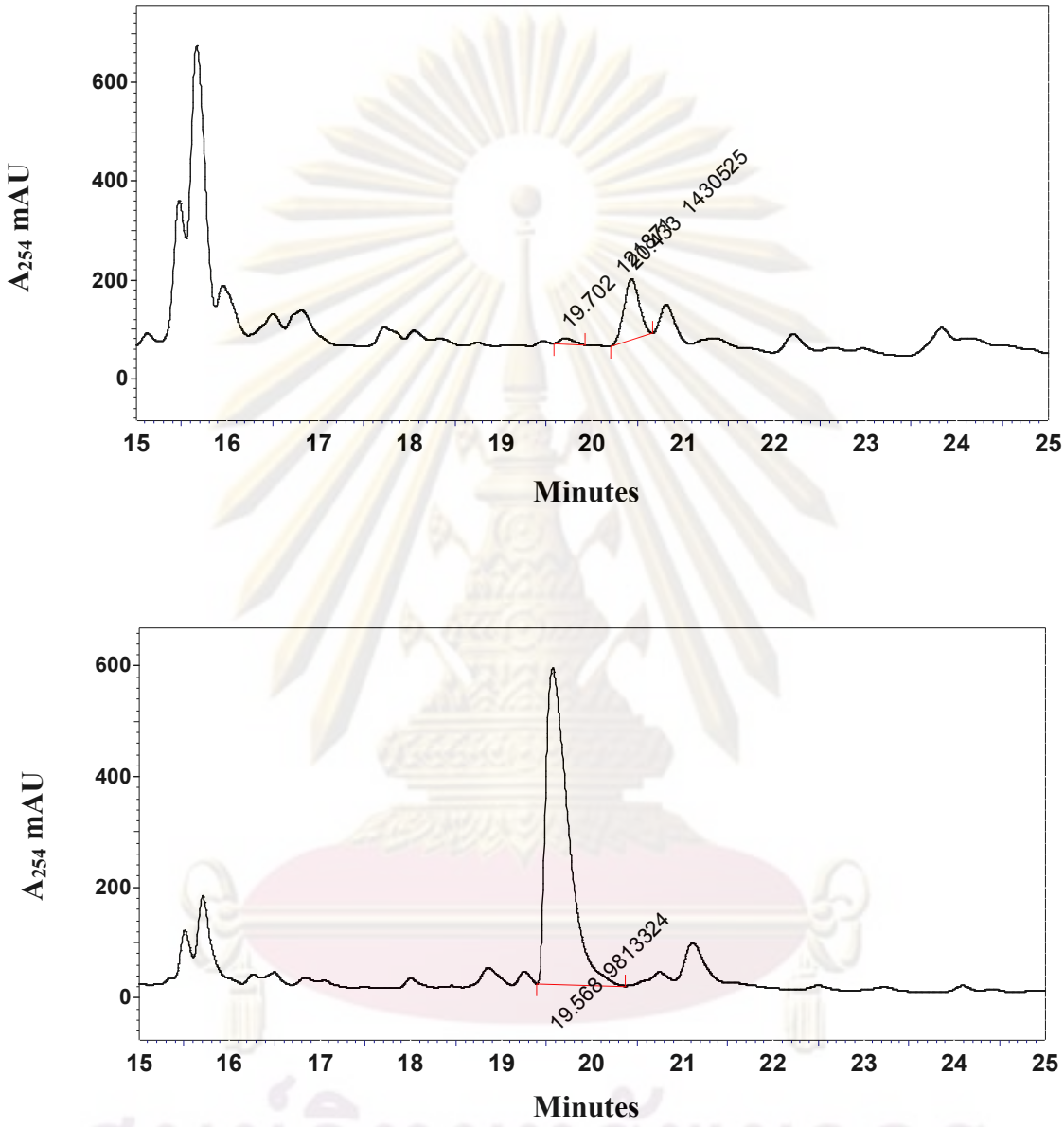

Figure F10 HPLC chromatograms of *in vitro* root of licorice cultured on 1/2B5+IBA 5 mgL^{-1} after 10 weeks (A); Spike chromatogram of 1/2B5+IBA 5 mgL^{-1} in standard glycyrrhizin 500 ppm mixture (B).

BIOGRAPHY

Mr. Wanchat Sawaengsak was born on May 5, 1984 in Bangkok, Thailand. He received Bachelor of Science in 2006 from Department of Biology, Faculty of Science, Mahidol University, Pyathai Campus, Thailand. He was admitted to the Master degree of Science in Biotechnology, Faculty of Science, Chulalongkorn University in 2007.

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