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Establishment and chemical analysis of hairy roots of Eucommia ulmoides

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ESTABLISHMENT AND CHEMICAL ANALYSIS OF HAIRY ROOTS OF *EUCOMMIA ULMOIDES*

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ABSTRACT

The bark of *Eucommia ulmoides* Oliver (Eucommiaceae) has been used over thousands of years as a folk remedy. Extracts from the bark exhibit multiple pharmacological functions, especially in lowering blood pressure. Geniposidic acid (GA), pinoresinol diglucoside (PG) and chlorogenic acid (CA) are three compounds isolated from the bark of the Eucommia tree, which display enormous bioactivities.

As a type of plant tissue culture, the hairy root culture system displays prospective application over traditional cell or callus cultures, which are characterized by rapid growth, and stable biochemical and genetic capacity. The present dissertation discusses the establishment and chemical analysis of *E. ulmoides* hairy roots.

After molecular identification, including PCR and Southern blotting, hairy roots were subjected to various physical and chemical treatments to investigate their impacts on growth and secondary metabolites. Results showed that light, initial pH, and culture volume did not affect the growth remarkably, while medium type, medium strength, sucrose concentration and auxin influenced the growth significantly. Biomass of *E. ulmodies* hairy roots increased 30% in modified culture conditions.

Initial pH only had effect on PG content, while culture volume showed some effect on CA content. Other factors demonstrated varied impacts on content and yield of secondary metabolites. Zero to 50 μM methyl jasmonic acid (MeJA) elicited PG synthesis but inhibited GA production. A low concentration of salicylic acid (SA) reduced secondary metabolic synthesis, while a high concentration of it accelerated the exudation of the metabolites into medium. Exposure to light tended to improve synthesis of secondary metabolites, especially GA and CA. In contrast to other sources of *E. ulmoides*, hairy roots could synthesize high amounts of secondary metabolites, even specific compounds.

In order to increase secondary metabolite production, *Vitreoscilla* hemoglobin (VHb) was introduced into *E. ulmoides* hairy root. Molecular characterization, i.e. PCR, Southern blotting and Northern blotting, confirmed the integration and expression of the VHb gene. The transformed hairy root showed improved growth and enhanced synthetic capacity of secondary metabolites.

This research will contribute to exploit a new approach to produce PG, GA and CA from the special tissue culture, hairy root culture system.

Keywords: chlorogenic acid; *Eucommia ulmoides*; geniposidic acid; hairy root; methyl jasmonate; pinoresinol diglucoside; salicylic acid; tissue culture; *Vitreoscilla* hemoglobin

CHAPTER 1. INTRODUCTION

Eucommia ulmoides Oliver (Eucommiaceae), known as Hardy Rubber tree in the U.S. and DuZhong in China, is native to the mountainous regions of China (Fig 1.1.). Due to its versatile application as traditional Chinese medicine, the species is now cultivated widely in Korea, Japan, North Europe and North America (Wang et al. 2003).

Fig 1.1. *E. ulmoides* grown at the LSU Agricultural Center's Burden Research Station in Baton Rouge

Reportedly, extracts from *E. ulmoides*, especially from the bark, exhibited various pharmacological functions, including lowering blood pressure, slowing aging, healing organs and strengthening muscles and bones (Kwan et al. 2003; Li et al. 1998). The iridoids, lignans, flavonoids and phenylpropanoids identified in *E. ulmoides* are the major constituents responsible for its biomedical properties. Pinoresinol diglucoside (PG, Fig 1.2.), one of the lignans in *E. ulmoides*, is considered to be the main hypotensive principle (Sih et al. 1976). Recent studies found that *Eucommia* bark extract standardized to PG significantly reduced blood pressure of hypertensive rats when administrated orally (Lang et al. 2005; Liu. 2004) and was safe and effective in a recently completed pilot human clinical trial conducted by our lab in Pennington Biomedical Center. Geniposidic acid (GA, Fig 1.3.), an iridoid glycoside

produced by *E. ulmoides*, displayed antimutagenic, antioxidant, antitumor, and radioprotective properties (Duke. 1992; Hsu et al. 1997; Ueda et al. 1991). Chlorogenic acid (CA, Fig 1.4.) or 3-caffeoylquinic acid, another active constituent of *E. ulmoides*, also demonstrated preventive effects regarding lipid peroxidation and elimination of hydroxyl free radicals (Yang et al. 2004).

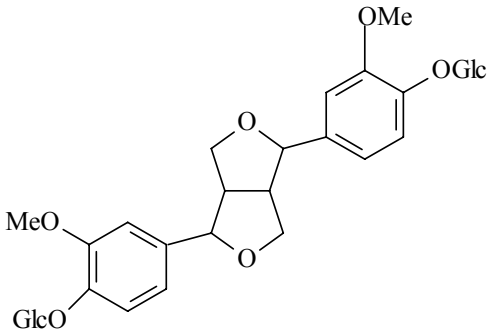


Fig 1.2. Chemical structure of pinoresinol diglucoside (PG)

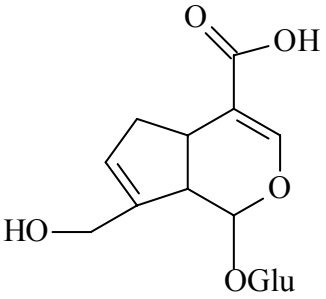


Fig 1.3. Chemical structure of geniposidic acid (GA)

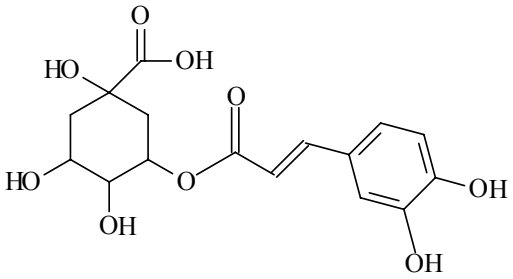


Fig 1.4. Chemical structure of chlorogenic acid (CA)

Use of Eucommia bark as tonics appears to be increasing despite the fact that present yield falls short in meeting the demand for raw materials (DeBosier. 1997). Relying on the conventional field production for these raw materials has many restrictions such as variations

of active principles due to influence of cultural practices, environmental stress, long growing to maturity intervals and post-harvest regeneration. In the current regime, harvesting bark itself is not hindering the supply. Although a none-destructive girdling method to harvest the tree bark has been developed and practiced, the debarking process still requires the trees to grow to certain sizes over several years and worse, often causes ringed surface necrosis. Moreover, the production of secondary metabolites in *E. ulmoides* is dependent on many factors, which include harvest time, post-harvest treatment, and production area. In addition, environmental factors, such as rainfall, temperature, soil acidity, and location, and genetic factors, including budding, leaf shape, and bark type are important factors to be considered in the regulation and synthesis of secondary compounds (Zhang et al. 1999).

Tissue and cell cultures such as callus and cell suspension cultural systems of *E. ulmoides* have already been established (Huang et al. 2003; Wang et al. 2003), and PG and CA were found to be synthesized in such systems (Asaumi et al. 1992; Tang et al. 2002). Compared to the mentioned cultures, an organ culture system such as hairy root culture has been used to produce secondary metabolites at comparable, or greater levels than those of the intact tree (Sevón 2002). Hairy root is obtained by transformation with *Agrobacterium rhizogenes*, which can grow on auxin free medium. More importantly, hairy root grows rapidly and is capable of sustaining biochemical and genetic stability (Azlan et al. 2002). They are also suitable for bioreactor systems (Wysokińska et al. 1998). Therefore, hairy root culture holds a greater promise for a viable industrial application. Unfortunately, this great system for producing secondary compounds has not been attempted or reported for Eucommia. The need for *E. ulmoides* bark and the prospect of short supply prompted the investigation of the hairy root culture of *E. ulmoides*. This study reports the establishment and chemical analysis of *E. ulmoides* hairy roots.

CHAPTER 2. ESTABLISHMENT OF HAIRY ROOT CULTURE SYSTTEM OF *EUCOMMIA ULMOIDES*

2.1. Background

Hairy root is a kind of plant disease caused by *Agrobacterium rhizogenes*, a Gram-negative soil bacterium. When the bacterium infects the plant, the T-DNA between TR and TL regions of the Ri-plasmid inside the bacterium is transferred and integrated into the nuclear genome of the host plant. The transformation process produces a valuable by-product, hairy root, which will form at or near the site of infection, in addition to opines to serve as specific food for the bacteria (Chilton et al. 1982). Hairy roots grow rapidly, show plagiotropic growth, and are highly branched on phytohormone-free medium.

It is well known that many plant secondary metabolites are important sources for production of pharmaceuticals, perfumes and food additives. The yields of secondary metabolites extracted from field grown plants vary widely and depend on many factors that are difficult to control. The production of secondary metabolites is often influenced by pests, climate or even political instability in regions where the plants are grown. Therefore, the biotechnological methods to produce certain metabolites in bioreactors seem to be an attractive alternative. Many efforts have been made to commercialize production of secondary metabolites from plant cell-culture in bioreactors but very few have achieved commercial success. One of the key obstacles to the success is that bioreactor design, operation, and control are very expensive and often the cost of the fixed expenses (depreciation, interest, and capital expenditures) account for over half of the manufacturing costs (Shanks et al. 1999). Another obstacle is that only products from endangered plants not amenable to agronomic cultivation will have prospective marketing potential. Unfortunately, although many important metabolites were found in cell-culture, those cell cultures usually have a strong tendency to be genetically and biochemically unstable and are often synthesized at very low levels of useful secondary metabolites. This low concentration of

certain secondary metabolites is largely due to developmental stages of these production cells, which divide and grow rapidly but without undergoing differentiation. The biosynthesis and accumulation of the secondary metabolites are exactly correlated with the extent of cellular differentiation (Kittipongpatana et al. 1998; Merkli et al. 1997; Rhodes et al. 1990; Verpoorte et al. 1987).

Compared with suspension cell culture, transformed hairy root is highly differentiated and can produce plant materials that are rich in secondary metabolites. This elevated level of secondary metabolites and the rapid growth of transfected hairy roots are just the features of a successful production model for useful phytochemicals. For example, cell suspension cultures of *Cinchona officinalis* 'Ledgeriana' were poor producers of quinoline alkaloids (less than 1 $\mu g\ g^{-1}$ DW), while hairy root of the same plant produced up to 50 $\mu g\ g^{-1}$ fresh weight (FW) quinoline alkaloids (Geerlings et al. 1999). The levels of the steroidal alkaloid solasodine were significantly higher in hairy root cultures than either callus or cell suspensions (Kittipongpatana et al. 1998), both of which are in the undifferentiated state of the development. And sometimes, hairy root can accumulate certain secondary metabolites that can not be found in wild-type root. For example, lawsone, a napthoquinone derivative, is restricted to the aerial parts and not the roots of wild-type henna; however, it is found in significant quantities in hairy root cultures (Bakkali et al. 1997). Currently, there is no report of *E. ulmoides* hairy roots. This chapter will discuss the establishment of the hairy culture system of *E. ulmoides*.

2.2 Objective

To establish the hairy root culture system of *E. ulmoides*.

2.3 Materials and Methods

2.3.1. Materials

Agrobacterium strain LBA 9402 was bestowed by Professor Zhibi Hu at Shanghai University of Traditional Chinese Medicine located in Shanghai of P.R. China, while other agrobacterium strains ATCC 31798, ATCC 15834, ATCC 39207, and ATCC 11325 were purchased from ATCC Company (Manassas, VA). Seeds of *E. ulmoides* were collected from trees grown at the LSU Agricultural Center's Burden Research Station in Baton Rouge.

2.3.2. In Vitro Germination

Seeds of *E. ulmoides* were germinated *in vitro* according to the following procedures. After removing the fruit coats, the seeds were immersed in water overnight at room temperature to allow imbibitions. The seeds were next surface-sterilized using the protocol as follows: 30 min in 1% (v/v) Clorox bleach (5.25% sodium hypochlorite) solution, 1 min in 70% (v/v) ethanol solution, and 30 min in 10% (v/v) bleach followed by 5 rinses of sterile water. The sterilized seeds were inoculated into half-strength MS basal medium. One month later, the germinated seedlings were subcultured in full-strength MS medium without supplemental plant growth regulators.

2.3.3. Hairy Root Induction and Culture

True leaves from *in vitro* germinated *E. ulmoides* seedlings were excised into 1 cm^2 pieces and used as explants for transformation. Five strains of *Agrobacterium rhizogenes*, LBA 9402, ATCC 31798, ATCC 15834, ATCC 39207, and ATCC 11325, were used to transfect the explants, respectively. The bacteria stored at -80ºC were first activated by inoculation on solid YMB media (1 g of yeast extract, 0.2 g of $MgSO_4 \cdot 7H_20$, 0.5 g of K_2HPO_4, 0.1 g of NaCl, and 10 g of mannitol per liter) and cultured at 28ºC for two days. Then the bacteria were transferred into liquid YMB media by using a sterile bacterial loop and cultured on an orbital shaker at 280 rpm and 28ºC overnight. Acetosyringone (50 μM)

was added to the bacterial culture media after that overnight culture to increase the frequency of hairy root induction (Giri 2001). After two days co-culture with different strains of *A. rhizogenes* on hormone-free MS basal media, the explants were washed five times with sterile water to remove superficial bacteria and then were transferred to MS basal medium containing cefotaxime (500 μg/ml) to further eliminate excess bacterial growth. One to two weeks later, when hairy roots initiated from the edges of the explants and grew to nearly 2 cm long, they were excised and subcultured onto solid MS medium for two months. Then they were transferred to liquid MS basal medium supplemented with 3% (w/v) sucrose and subcultured every four weeks.

2.3.4. PCR and Southern Blotting Analysis

Genomic DNA was extracted from fresh hairy roots and purified according to acetyl trimethyl ammonium bromide (CTAB) method (Sambrook et al. 1989). For PCR analysis, a pair of primers, 5'-GATATATGCCAAATTTACACTAG-3' and 5'-GTTAACAAAGTAGGAAACAGG-3', was used to amplify a 557 bp fragment of *rolC* gene. The 20 μl PCR reaction mixture contained 1 unit of *Taq* DNA polymerase (Promega, Madison, WI), 0.5 μl of 10 mM dNTPs, 2 μl of 10×PCR buffer, 1.6 μl of 25 mM $MgCl_2$, 2 μl of each primer (5 μM) and 100 ng of genomic DNA. PCR was conducted under the following conditions: 94 ºC pre-denaturation for 5 min; 94 ºC denaturation for 0.5 min, 56 ºC annealing for 0.5 min, 72 ºC extending 1 min for 30 cycles; followed by an extra extending cycle of 7 min. Finally, 5 μl of each amplified product was detected by electrophoresis on 1.2% agarose gel.

For Southern blotting analysis, a total of 15 μg genomic DNA was digested with the restriction endonucleases *Bam*HI and *Eco*RV overnight and transferred onto a Hybond^{TM} N^+ nylon membrane (Amersham, Piscataway, NJ), followed by UV cross-linking to fix the DNA on the membrane. A DNA fragment of *rol*C gene, ca. 500 bp, was used as the template for

randomized primer labeling and hybridized with the blot according to the manual of DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Indianapolis, IN). Signals were visualized by chemical and immunological detection methods within the same kit.

2.3.5. Growth Pattern

One of the most vigorously growing hairy root lines was chosen for growth pattern study. The hairy roots, ca. 0.5 g in fresh weight, were inoculated into 125 ml Erlenmeyer flasks containing 50 ml MS liquid media and cultured continuously on an orbital shaker in the dark at 100 rpm and 25ºC for eight weeks. Each week five flasks were harvested for the analysis of both fresh and dry weight. The experiments were repeated twice. All the data were collected and subjected to ANOVA analysis by using the MIXED program of SAS software.

2.4. Results

2.4.1. In Vitro Germination of *E. ulmoides*

After one month of germination, seedlings of *E. ulmoides* were transferred into baby food jars with MS medium (Fig 2.4.1). Three to four pairs of true leaves were grown in *E. ulmoides* seedlings.

2.4.2. Induction of Hairy Roots

After co-cultured with agrobacteria for one week in Petri dishes with MS medium containing cefotaxime, hairy roots began to develop from the brink of the true leaf discs (Fig 2.4.2). When they grew to nearly 2 cm in length, hairy roots were excised from explants and transferred to new MS medium without any auxin. After one month, roots that did not survive were normal roots, whereas roots that survived were true hairy roots. Hairy roots are capable of synthesizing endogenous auxin, thus require no supplemental auxin. The autotrophy in auxin production reflects the expression of genes within the T-DNA of the Ri plasmid from *A.*

rhizogenes. The survived roots displayed the typical phenotypes of hairy roots, such as rapid growth, highly branched, plagiotropism, and auxin autotrophy.

Fig 2.4.1. *E. ulmoides* seedlings germinated for one month from seeds collected from trees grown at the LSU Agricultural Center's Burden Research Station in Baton Rouge.

Fig 2.4.2. Hairy roots induced from true leaves of *E. ulmoides*

2.4.3. Frequency of Transformation of Agrobacteria

The strains of *A. rhizogenes* varied greatly in their ability to induce hairy roots, and their frequency of transformation ranged from 0% to 100%. LBA9402 had the highest induction frequency, while ATCC 11325 did not infect the explants at all. The infectivity of other stains fell between the two strains (Table 2.4.3).

Table 2.4.3. Comparison of different strains of *Agrobacterium rhizogenes* in their ability to induce hairy roots on *Eucommia* leaf explants. Definition: frequency of transformation is calculated by the number of explants induced with hairy roots dividing total number (n=30) of explants used for transformation.

Bacterial strain	Frequency of transformation (%)
LBA 9402	100.0
ATCC 15834	80.0
ATCC 31798	16.7
ATCC 39207	70.0
ATCC 11325	0

2.4.4. Molecular Identification

To confirm the integration of T-DNA from the soil bacteria into the hairy root genomic DNA, DNA from hairy roots were subjected to PCR and Southern blotting analysis, respectively. PCR was used to demonstrate that the T-DNA from the Ri plasmid of *A. rhizogenes* was present in *E. ulmoides* hairy roots. The PCR results showed that all the hairy root lines contained *rol*C gene which was a part of T-DNA of Ri plasmid of *A. rhizogenes* (Fig 2.4.4.1). Southern blotting analysis further corroborated PCR results and confirmed the stable incorporation of T-DNA into *E. ulmoides* genomes (Fig 2.4.4.2).

2.4.5. Growth Pattern of Eucommia Hairy Root

The growth pattern of *E. ulmoides* hairy root was a typical sigmoid curve. As shown in Fig 2.4.5, the growth of hairy root in the first three weeks was a lag phase, followed by a three weeks of rapid growth phase, i.e. log phase, and then ended at a stationary phase. The biomass yield increased by 7.4 fold in six weeks (based on fresh weight change) and attained 0.75 g dry weight per flask. The growth pattern of *E. ulmoides* hairy root in 125 ml

Erlenmeyer flasks containing 50 ml of MS liquid media could be fit to a Logistic Model (eq 1) for both fresh and dry weights as follows:

$$Y = \frac{a}{1 + be^{-cx}} \qquad \text{[eq 1]}$$

where Y stands for yield (g) and X stands for time (week). The values of coefficients were listed in Table 2.4.5. The predicted time for yield doubling of hairy root is 1.88 weeks according to the Logistic Model.

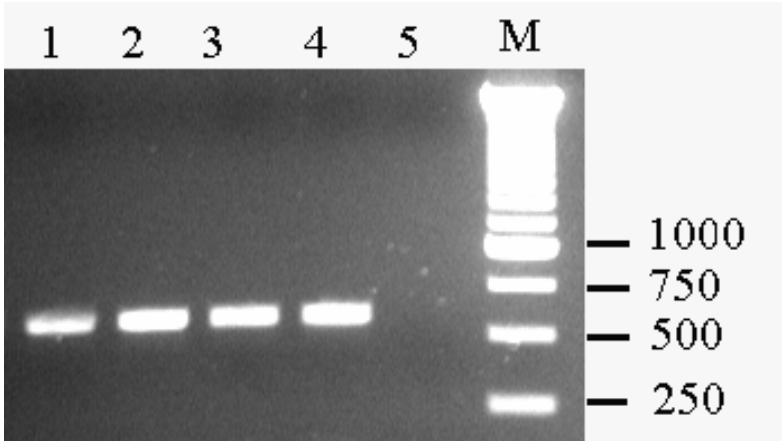


Fig 2.4.4.1. PCR analysis of genomic DNA from selective hairy root lines and seedlings of *E. ulmoides* to amplify a 557 bp fragment of *rol*C gene. Lanes: 1-4 hairy root lines; 5 seedlings; M 250 bp DNA Ladder (Invitrogen, Carlsbad, CA) and some sizes of fragments were labeled on the right.

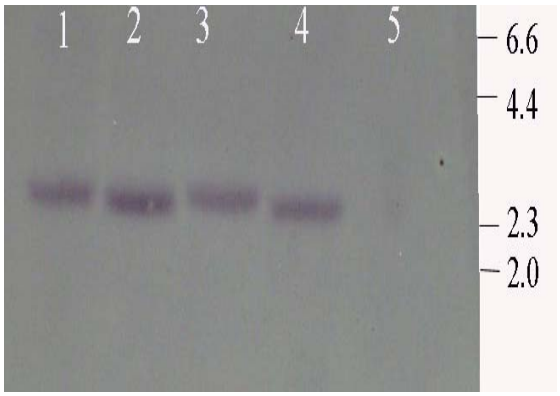


Fig 2.4.4.2. Southern-blot hybridization analysis of genomic DNA isolated from selective hairy root lines and seedlings of *E. ulmoides*. DNA samples (15 μg) were digested with *Bam*HI and *Eco*RV, and hybridized with digoxigenin-labeled *rol*C gene probe. Lanes: 1-4 hairy root lines; 5 seedlings. Fragment sizes (kb) on the right correspond to a λ/*Hin*dIII DNA marker.

2.5. Discussions

Approximately 25% of contemporary materia medica is derived from plants (Kutchan 1995). Unfortunately, content of most active components within plants is very low since they are secondary metabolites, which are stimulated as a defense response to pathogens or herbivores. Moreover, synthesis of secondary metabolites is influenced by environmental conditions and varied at different stages of plant development. For the past decades, plant cell or organ cultures have caused much attention in production of secondary metabolites. However, the yield of such compounds is often low and unstable in callus or cell cultures. In contrast, hairy root culture shows greater promise for production of these active compounds at levels that are comparable to, or greater than the intact plants (Sevón 2002). The difference of degree of organization of cell structures is one of the possible reasons. Furthermore, hairy roots grow very fast and can maintain genetic stability over subcultures. Hairy roots have been induced in over hundreds of species. But the present paper is the first report on *E. ulmoides* hairy roots. Hairy roots of *E. ulmoides* showed the typical phenotype as hairy roots from other species, including auxin autotrophy, rapid growth and high branching. The growth pattern of *E. ulmoides* hairy roots was a typical sigmoid shaped curve, which could fit to a Logistic Model that could be used as a guide for harvesting to get maximal biomass accumulation.

Factors including Agrobacterium strains, age and differentiation status of plant tissue, co-culture time, and activation factor, i.e. acetosyringone, are often considered in order to increase frequency of transformation (Luo et al. 2004). Five strains of *A. rhizogenes* were used in present study. LBA 9402, ATCC 15834, ATCC 31798 (related to A4) and ATCC 39207 (spontaneous mutant derivative of TR 105) are grouped into Agropine-type strains, which are the most virulent, thus are among the first choices in the establishment of hairy root cultures (Sevón 2002). ATCC 11325 was suggested to be placed into the *A. tumefaciens*

group due to nopaline production. However, it still was successful to transform gymnosperm species (Buer et al. 1998). The different infectivity of *A. rhizogenes* might be due to different host susceptibility of the strains used (Drewes et al. 1995).

Acetosyringone is one of the phenolic inducers of the virulence genes of agrobacteria. The addition of the chemical into cultures of *A. tumefaciens* was found to increase transformation rate in *Arabidopsis thaliana* and *Atropa belladonna* (Mathews et al. 1990; Sheikholeslam et al. 1987). Studies also showed the promoting effect of acetosyringone on *A. rhizogenes* mediated transformation in *Brassica napus* (Boulter et al. 1990), *Salvia miltiorrhiza* (Hu et al. 1993), and *Artemisia annua* (Giri et al. 2001). Therefore, acetosyringone was added into bacterial culture media to increase the frequency of hairy root induction from *E. ulmoides* true leaves.

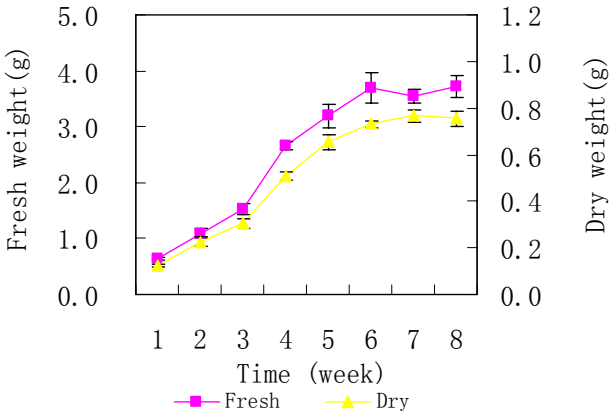


Fig 2.4.5. Fresh and dry weight change of *E. ulmoides* hairy roots cultured in 125 ml Erlenmeyer flasks containing 50 ml of MS liquid media during eight weeks. Each data point is the mean of ten repetitions. The vertical bars represent standard error.

Table 2.4.5. The coefficients in the Logistic Model of growth for both fresh and dry weight of Eucommia hairy roots.

Coefficient	For fresh weight	For dry weight
a	4.2587	0.8758
b	13.6288	13.3764
c	0.7592	0.7161
Standard error	0.3056	0.0424
Correlation coefficient	0.9690	0.9844

2.6. Summary

Induction of hairy roots was performed with five strains of *Agrobacterium rhizogenes*, including LBA 9402, ATCC 31798, ATCC 15834, ATCC 39207, and ATCC 11325, and efficiency of the induction was evaluated using true-leaf explants. LBA 9402 showed the highest frequency of transformation, while ATCC 11325 did not infect the explants. Molecular identifications (i.e., PCR and Southern blotting) confirmed the integration of T-DNA from the soil bacteria into hairy root genomes. The growth pattern of *E. ulmoides* hairy root was a typical sigmoid-shape curve, which attained its stationary phase in six weeks with an accumulative yield of 7.4 fold.

CHAPTER 3. INFLUENCE OF VARIOUS CULTURE CONDITIONS ON THE GROWTH AND CHEMICAL COMPONENTS OF *EUCOMMIA ULMOIDES* HARIY ROOTS

3.1. Background

In general, in vitro plant cell and organ cultures for production of secondary metabolites often meet with the bottleneck of low yield. Optimizing the composition of nutrients for hairy root cultures is critical to gain high production of secondary metabolites. Factors such as the carbon source and its concentration, ionic concentration of the medium, pH of the medium, light, phytohormones, temperature and inoculum, are known to influence the growth and secondary metabolism of hairy roots (Arroo et al. 1995; Bhadra et al. 1995; Christen et al. 1992; Morgan et al. 2000; Rhodes et al. 1994; Toivonen et al. 1992; Vanhala et al. 1998;). Heavy metal ions, concentration of phosphate, nitrate and ammonia are also well studied (Christen et al. 1992; Payne et al. 1987; Sevon 2002; Toivonen et al. 1991). Addition of auxin and elicitors often increased the levels of secondary metabolites (Dymov et al. 1997; Pittaalvarez et al. 1998; Rijhwani et al. 1998; Singh et al. 1998; Vanhala et al. 1998).

The use of biotic or abiotic elicitors is the common strategy employed to enhance productivity. Salicylate and jasmonate are both endogenous signal molecules that are elicited by plant as a defense response in order to resist pathogens and herbivores. These two compounds have been reported to be effective in inducing the synthesis of secondary metabolites in transformed roots of many species (e.g., valepotriates in *Valerianella locusta*, Kittipongpatana et al. 2002) scopolamine and hyoscyamine in *Brugmansia candida* (Pitta-Alvarez et al. 2000), and diacetylenes in *Tanacetum parthenium* (Stojakowska et al. 2002).

Due to so many factors involved in hairy roots cultures, the culture conditions should be investigated separately for each species. As *E. ulmoides* hairy roots have been induced in our lab and PG, GA and CA are found to be synthesized, the next reasonable step should focus on the investigation of an ideal culture condition to achieve as high a production of secondary

metabolites as possible. This chapter will discuss medium type, initial pH of medium, light condition, medium strength, sucrose concentration, culture volume, and elicitors on the growth and secondary metabolites of *E. ulmoides*.

3.2 Objective

To investigate various culture conditons on the growth and secondary metabolites of *E. ulmoides* hairy roots.

3.3. Materials and Methods

3.3.1. Hairy Root Subculture

After initiating and growing about 2 cm long, hairy roots were excised from explants and subcultured on MS solid medium supplemented with 3% sucrose. Cefotaxime (500 μg/ml) was added to the medium to eliminate or kill the remaining *A. rhizogenes*. After several rounds of subculture, the disinfected hairy roots were subcultured in MS liquid medium on an orbital shaker in the dark at 100 rpm and 25ºC. Then molecular identification, i.e. PCR and Southern blotting, was conducted to confirm the integration of bacterial T-DNA into hairy roots. One of the positive and most vigorously growing hairy roots was selected for further study.

3.3.2. Influences of Various Culture Condition on the Growth of Hairy Roots

The *E. ulmoidies* hairy roots, ca. 0.5 g in fresh weight, were inoculated into 125 ml Erlenmeyer flasks containing 50 ml MS liquid media supplemented with 3% sucrose and cultured continuously on an orbital shaker in the dark at 100 rpm and 25ºC for four weeks unless mentioned otherwise. After harvesting, the fresh weight and dry weight of hairy roots were measured separately. To reduce experimental error due to different growth patterns of hairy roots in each flask for inoculation, complete block design was used in all the experiments. Hairy roots inoculated from the same flask were set as one block. Each treatment was applied once in one block. At least four blocks were used to test each single

factorial effect. Each single factorial experiment was carried out in the same culture chamber and on the same shaker to avoid systematic error maximally.

3.3.2.1. Light and Medium Type

Two light conditions, light and dark, and three types of media, MS (Murashige and Skoog.1962), WPM (Lloyd and McCown. 1980) and B5 (Gamborg and Eveleigh. 1968), were tested together in one experiment. The light condition was set as a 16 h photoperiod (100 μmol m^{-2} s^{-1}), while the dark condition was made by wrapping the flasks with foil completely. The three types of prepared media were purchased from PhytoTechnology Laboratories directly. For B5 medium, 3.21 g of powder was used to prepare 1 L of medium. For MS medium, 4.43 g of powder was used to prepare 1 L of medium. For WPM mediu, 2.41 g of powder was used to prepare 1 L of medium. All the media were prepared using deioned water.

3.3.2.2. Initial Medium pH

Six initial medium pH conditions, from 4.0 to 7.0 at an interval of 0.6, were tested. All the media were regulated to required pH point prior to autoclave. pH values of the media were not monitored during culture period.

3.3.2.3. Medium Strength

Four medium strengths, 0.25, 0.5, 1 and 1.5, of MS medium were tested. Sucrose concentration was also changed at the same ratio.

3.3.2.4. Salt Strength and Sucrose Concentration

Three salt strengths of MS medium, 0.5, 1 and 1.5, and three sucrose concentrations, 1.5%, 3.0% and 4.5%, were tested together.

3.3.2.5. Culture Volume

Hairy roots were inoculated into four different sized flasks, i.e. 125 ml, 250 ml, 500 ml and 1000 ml, at the same medium/flask size ratio.

3.3.2.6. Auxin

Three types of auxins, IBA, NAA, and 2,4-D, each at two concentration levels, 1 μM and 10 μM, and one control without auxin were studied. All the auxins were added to the media and then subjected to autoclave.

3.3.3. Growth Pattern Comparison

When all the single factors were tested, the best single condition was selected individually and combined to form a new modified culture condition. Growth pattern of *E. ulmoides* hairy roots was investigated in the modified culture condition and compared with that in MS basal medium. About 0.5 g of the fresh hairy roots were inoculated into 125 ml Erlenmeyer flasks containing 50 ml new liquid media and cultured continuously on an orbital shaker at 100 rpm and 25ºC for six weeks. Each week five flasks were harvested and both fresh and dry weight were measured. Meanwhile, chemical components in the hairy roots were analyzed by HPLC.

3.3.4. Elicitation

Stock solution of SA (Sigma, St. Louis, MO) was prepared by dissolving SA in appropriate volume of 95% ethanol. Stock solution of MeJA was purchased from PhytoTechnology Laboratories (Shawnee Mission, KS). After cultured for four weeks, hairy roots were treated with series concentrations of MeJA (0, 0.1, 1, 10, 50, and 100 μM) and SA (0, 0.1, 0.5, 1, and 5 mM), respectively. As ethanol was used as the solvent for SA, treatment with only ethanol was added as a vehicle control for SA treatment group. To reduce experimental error due to different growth pattern of hairy roots in each flask for inoculation, complete block design was used in all the experiments. Hairy roots inoculated from the same flask were set as one block. Each treatment was applied once in one block. Four blocks were used, i.e. each treatment had four replicates.

3.3.5. Chemical Extraction and HPLC Analysis

After harvesting, hairy roots were freeze-dried immediately and ground into powder. For GA and PG analyzes, approximately 0.1 g of each sample was extracted with 1.5 ml 70% (v/v) methanol for three days. Then the extracts were subjected to centrifuge at 12,000 rpm. The supernatant was collected and blown to dry. The extraction procedure was conducted twice and the dried extracts were combined, dissolved in 1 ml distilled water and purified by C18 SPE column. The eluent of methanol was collected, dried, followed by dissolving in 0.3 ml 30% (v/v) methanol and filtered through a 0.2 μm syringe filter prior to HPLC analysis. For CA analysis, about 50 mg of each sample was extracted twice with 1 ml deionized water in a boiling water-bath for 1 hr. The extracts were then subjected to centrifuge at 12, 000 rpm. The supernatant was collected and freeze-dried. The freeze-dried extracts were then dissolved in 250 μl sterile water and filtered through 0.2 μm syringe filter before HPLC analysis.

HPLC analysis was conducted on a Waters 600 system with a 717 autosampler and a 2996 photodiode array detector at ambient temperature. The system was computer controlled and analyzed with the Empower software system. Separation was carried out using a Symmetry C18 column (5.0 μm, 150×4.6 mm I.D.) with a guard cartridge (5.0 μm, 20×3.9 mm I.D.). For GA and PG analyses, the mobile phase was set as follows: 0～10 min, MeOH-0.15% acetic acid/10:90; 10～40 min, gradient from MeOH-0.15% acetic acid/ 10:90 to 40:60; 40～50 min, MeOH. The flow rate was 1.0 ml/min and detection wavelength was set at 236 nm for GA and 226 nm for PG. The retention time of GA was 12.1 minute and that of PG was 37.8 minute. The analysis of CA was performed with a mixture of 0.15% (v/v) acetic acid and acetonitrile (92:8) as the mobile phase at a flow rate of 1 ml min^{-1}. The detection wavelength of CA was 325 nm with retention time at 11.2 min. The calibration curve for GA is as follows: Y=1229325*X-36276 with R^2=0.99990 and linear range: 0.315~ 1.575 μg. The calibration curve for PG is as follows: Y= 1536588*X -20592 with R^2=0.99995 and linear

range: 0.180~0.900 μg. For CA, the calibration curve is Y=4263632*X -411363 with R^2=0.99826 and linear range: 0.165~2.0625μg. For all the calibration curves, X represents amount (μg) and Y stands for peak area.

3.3.6. Statistic Analysis

All the data were collected and subjected to ANOVA analysis by using the MIXED program of SAS software. Tukey adjustment was used for all pairwise comparisons.

3.4. Results

3.4.1. Influence of Culture Condition on the Growth of Hairy Roots

3.4.1.1. Medium Type

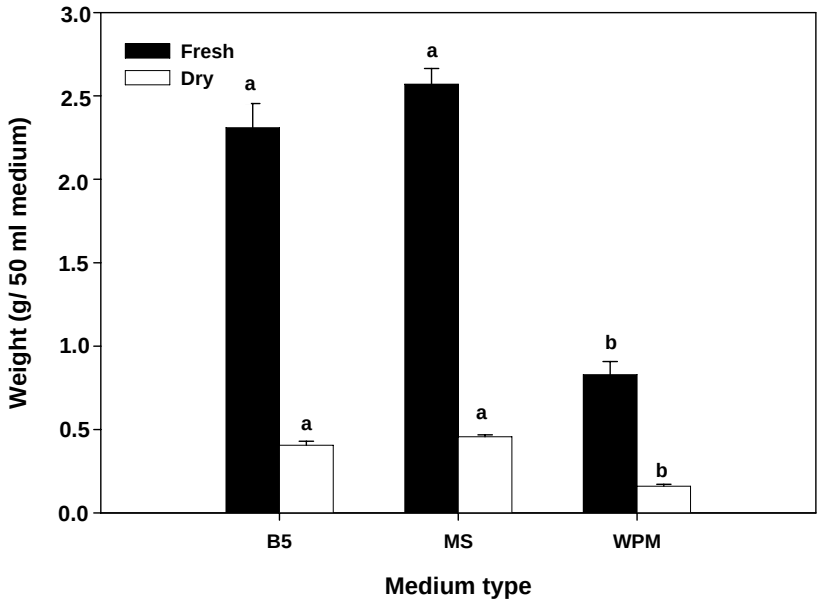


Fig 3.4.1.1. The effect of medium type on the growth of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated ten times (n=10). Significant differences between treatments are labeled by different small letters (α = 0.05).

Medium types displayed significant effect on both fresh and dry weight of *E. ulmolides* hairy roots (Fig 3.4.1.1.). WPM medium was the weakest medium for the growth of *E. ulmodies* hairy roots. Both the fresh and dry masses of hairy roots grown in WPM medium were significant lower than that in B5 and MS medium. MS medium was the best

medium among the three media, in which the dry mass of hairy roots attained 0.46 g per flask, but did not differ from that in B5 medium.

3.4.1.2. Light Condition

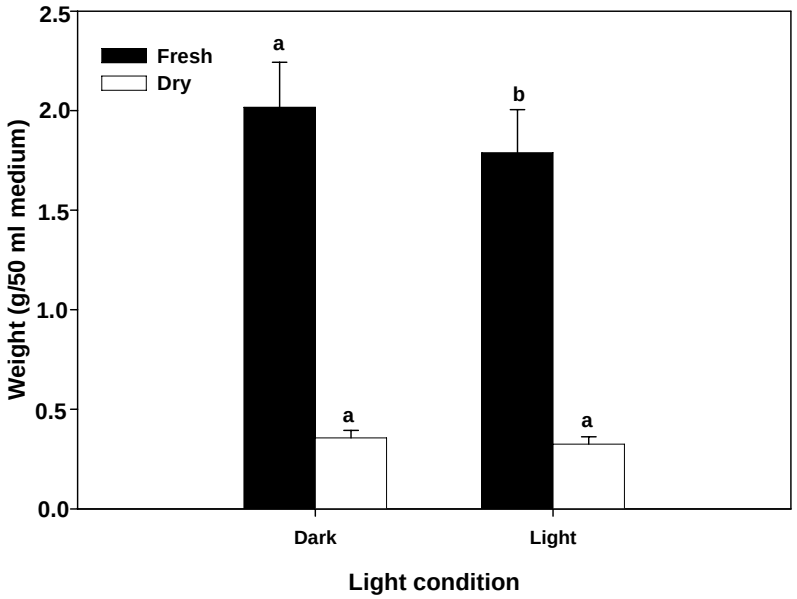


Fig 3.4.1.2. The effect of light condition on the growth of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated fifteen times (n=15). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

Light condition did not influence the dry mass accumulation of *E. ulmoides* hairy roots, although it affected the fresh mass of hairy roots (Fig 3.4.1.2). Hairy roots grown in the dark did produce more biomass yield but not significantly so from that in light condition. No significant interaction between medium type and light condition was found.

3.4.1.3. Initial pH

Initial pH did not affect the growth of hairy roots (Fig 3.4.1.3). Hairy roots grown in all the six tested initial pH displayed similar biomass accumulation in four weeks, either fresh or dry mass. Since pH is one of the important factors involved in many metabolic pathways, such as respiration, ion-transportation, and water-absorption, *E. ulmoides* hairy roots must have strong ability to adapt to and /or regulate various pH ranges.

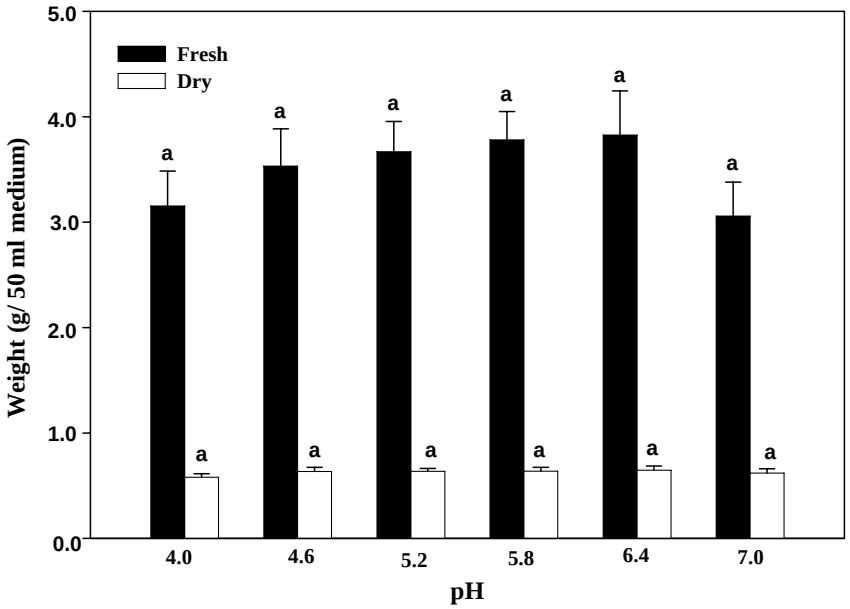
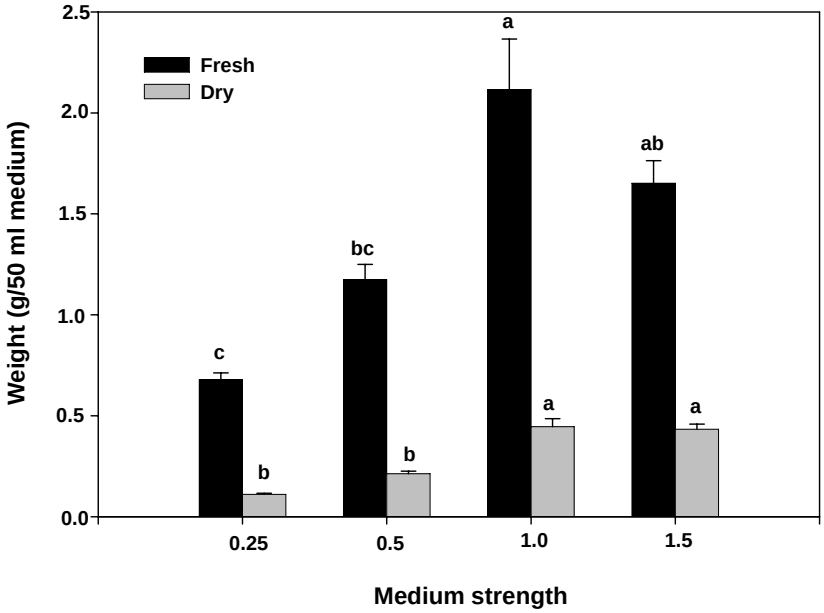


Fig 3.4.1.3. The effect of pH on the growth of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated five times (n=5). Significant differences between treatments are labeled by different small letters (α = 0.05).

3.4.1.4. Medium Strength

Medium strength had great effects on the growth of *E. ulmoides* hairy roots (Fig 3.4.1.4.1 and 3.4.1.4.2). Significant differences were found among the treatments of different medium strengths on both fresh and dry mass accumulation of hairy roots. As the medium strength decreased, biomass production of hairy roots reduced remarkably, especially when the concentration of sucrose decreased simultaneously. But when the strength of medium increased from 1.0 to 1.5, biomass accumulation also seemed to decrease but not significantly. When the concentration of sucrose reduced from 1.0 to 0.5 and 0.25 at the same ratio with medium strength, the biomass yield decreased dramatically and almost at the same decreasing ratio. As only the basal medium strength changed, i.e. the concentration of sucrose did not alter, biomass of hairy roots did not change much, although significant difference was found between 1.0 and 0.5.

Fig 3.4.1.4.1. The effect of medium strength on the growth of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated four times (n=4). Significant differences between treatments are labeled by different letters (α = 0.05).



3.4.1.5. Sucrose Concentration

Sucrose percentage in the medium influenced the growth of *E. ulmoides* hairy roots significantly (Fig 3.4.1.5). As sucrose percentage increased, biomass also accumulated accordingly. The highest biomass accumulation of *E. ulmoides* hairy roots was obtained in

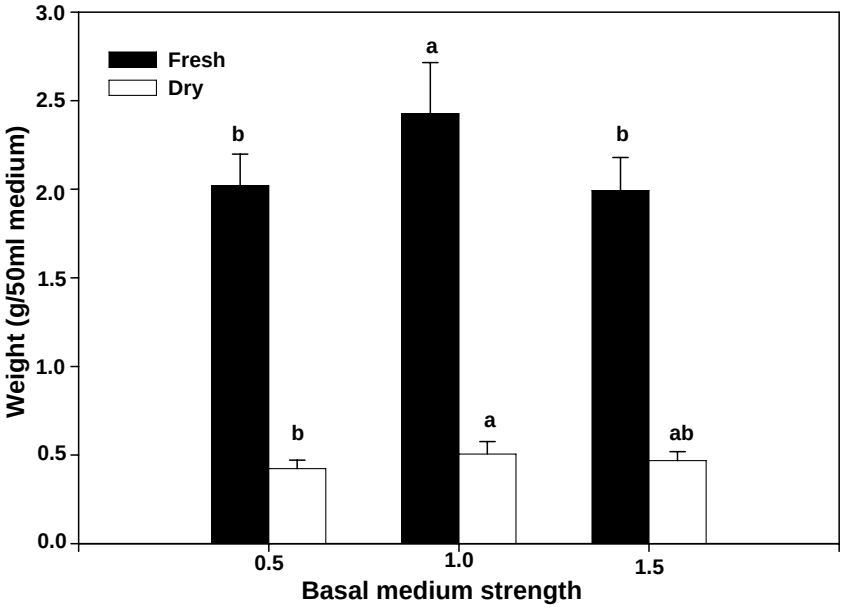


Fig 3.4.1.4.2. The effect of basal medium strength on the growth of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated four times (n=4). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

medium with 4.5% sucrose. No significant interaction between basal medium strength and sucrose concentration was found.

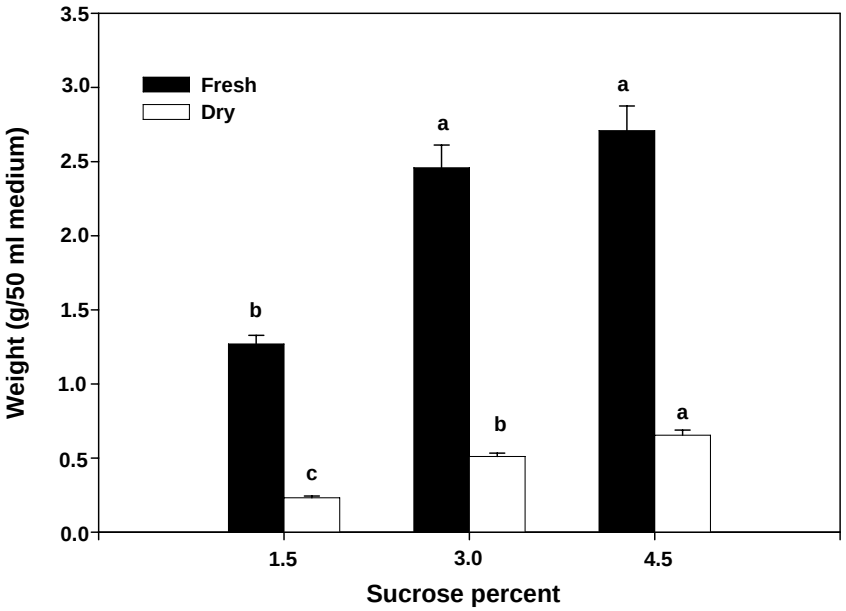


Fig 3.4.1.5. The effect of sucrose concentration on the growth of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated four times (n=4). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

3.4.1.6. Culture Volume

Flask size did not affect growth rate significantly (Fig 3.4.1.6). No significant difference among the four different sized flasks, i.e. 125 ml, 250 ml, 500 ml and 1000 ml, on the growth of hairy roots was found. However, hairy roots cultured in 250 ml flasks showed the highest biomass yield, followed by that in 1000 ml flasks. The results agreed with the study that the growth rate of hairy roots did not increase linearly with the enlargement of culture volume (Du et al. 2003).

3.4.1.7. Auxin

Auxins did not accelerate the growth of hairy roots too much (Fig 3.4.1.7.1). Conversely, as the concentration of auxins increased from 1 μM to 10 μM, the growth of hairy roots was inhibited to some extent. All three auxins used displayed the same inhibition pattern. One μM IBA increased biomass of hairy roots a little but not significantly different

from that without auxin. 2,4-D inhibited the growth of hairy roots remarkably. Furthermore, hairy roots in medium supplemented with 2, 4-D or high concentrations of IBA or NAA became callused and differed from normal hairy roots in morphology (Fig 3.4.1.7.2).

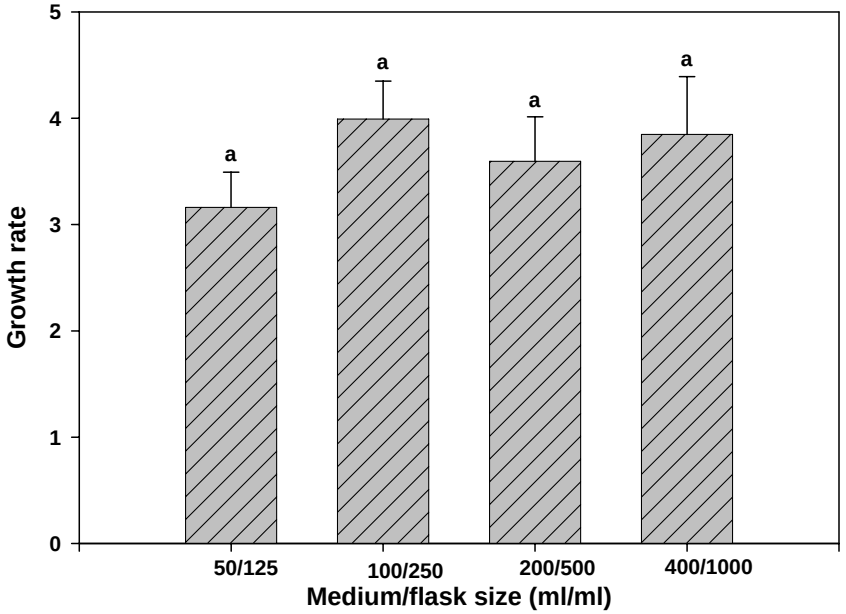


Fig 3.4.1.6. The effect of culture volume on the growth of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated three times (n=3). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

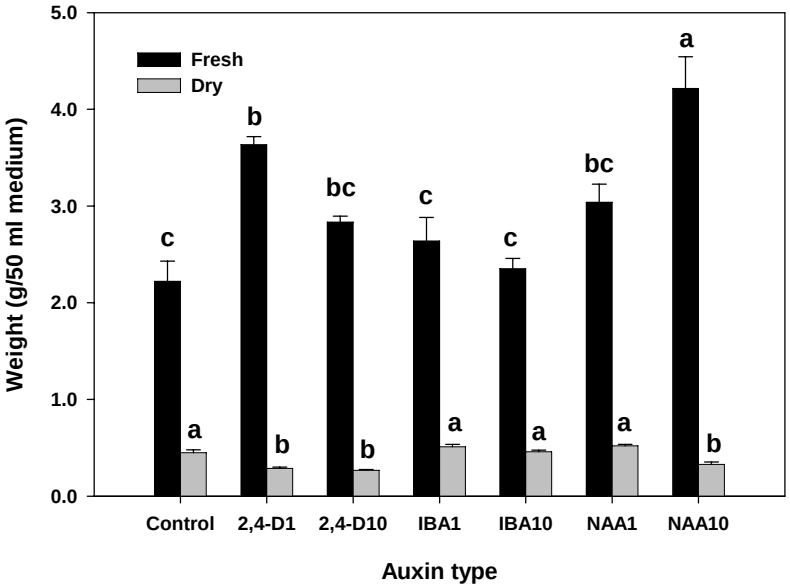


Fig 3.4.1.7.1 The effect of auxin type on the growth of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated four times (n=4). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

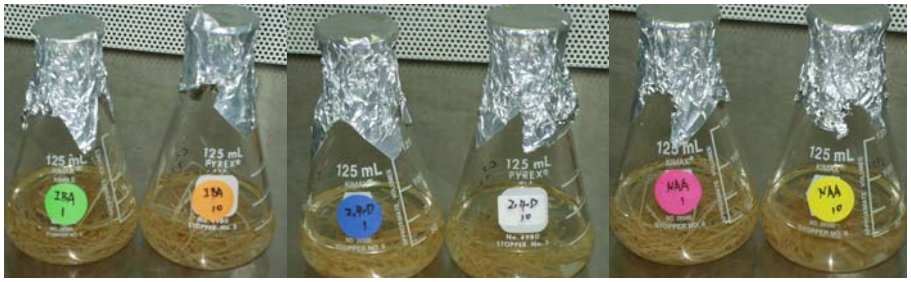


Fig 3.4.1.7.2 The effects of auxin on the phenotype of *E. ulmoides* hairy roots.

3.4.1.8. Hairy Root Lines

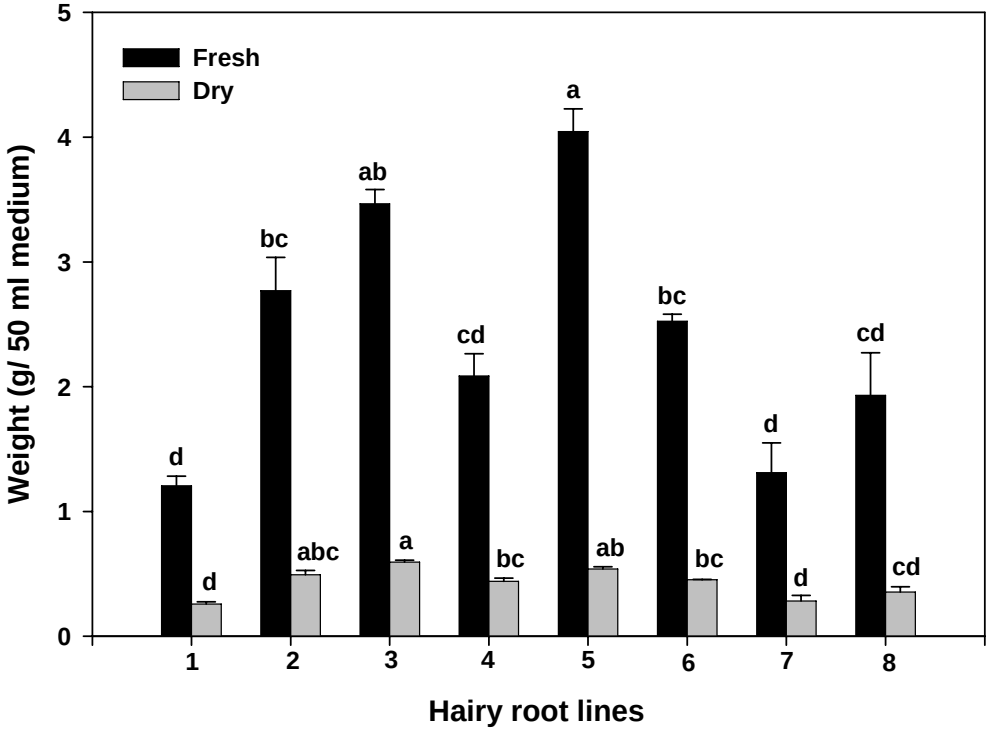


Fig 3.4.1.8 Comparison of fresh and dry weight of different *E. ulmoides* hairy root lines cultured in 50 ml liquid MS medium for four weeks. Vertical bars represent standard error. Each treatment is replicated three times (n=3). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

Hairy root lines showed varied fresh and dry mass accumulation patterns (Fig 3.4.1.8). In four weeks, dry mass of HRL-3 attained 0.59 g, which was 2.3-fold than that of HRL-1.

3.4.2.1. Comparison of Growth Pattern

The growth patterns of *E. ulmoides* hairy roots followed a typical sigmoid curve. As shown in Fig 3.4.2.1, the growth of hairy root over the first three weeks was lag phase, followed by another three weeks rapid growth, i.e. log phase, and then ended by a stationary

phase. The dry mass increased 7.4 fold in six weeks (estimated by fresh weight change) and attained 0.75 g dry weight per flask in MS basal medium. When cultured in the improved culture conditions based on all the single factorial experiments, i.e. in pH 5.8 MS medium supplemented with 1 μM IBA and 4.5% sucrose in dark conditions, *E. ulmoides* hairy roots showed similar growth pattern. Although the fresh mass of hairy roots in improved culture conditions seemed to decrease when compared to that in standard MS basal medium, the dry mass of hairy roots did increase by almost 30% in six weeks and attained 0.96 g per flask.

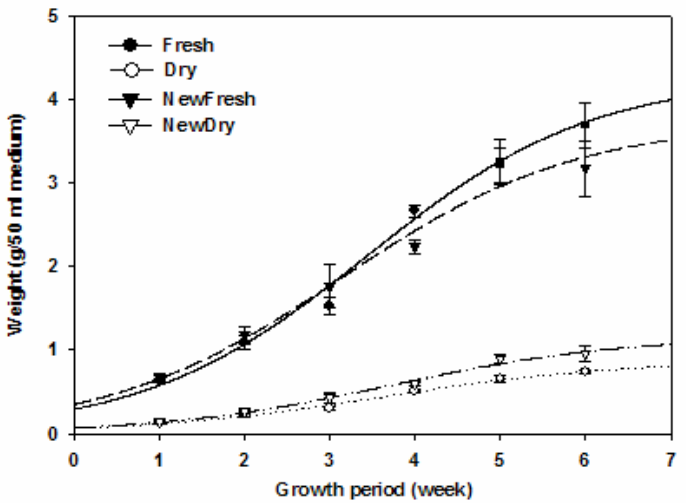


Fig 3.4.2.1 Comparison of growth pattern of *E. ulmoides* hairy roots in modified and MS basal medium.

3.4.3. Chemical Analysis

3.4.3.1 Impact of Culture Conditions on Chemical Component Content and Yield

3.4.3.1.1. Medium Type

Medium type affects both the content and yield of the three compounds (Fig 3.4.3.1.1.1-2). Hairy roots cultured in WPM medium produced the highest content of GA but gave the lowest yield of GA. Content of GA of hairy roots in MS medium was the lowest but the yield of GA was not significantly different from the other two types of media. Although the content of GA was not different from the other two remarkably, the yield of GA of hairy roots in B5 medium was the highest. For PG, hairy roots in WPM had the highest content but the highest yield was produced in MS medium and was significantly different from the other

two. For CA production of hairy roots, B5 medium was the best for both content and yield. Although CA content of hairy roots in MS medium was markedly lower than that in B5 medium, the yield of CA in it did not significantly differ from that in B5 medium. WPM was the weakest medium for both content and yield of CA. As a result, B5 medium was the best for GA and CA production, while MS medium was the best for PG synthesis in terms of maximal yield accumulation.

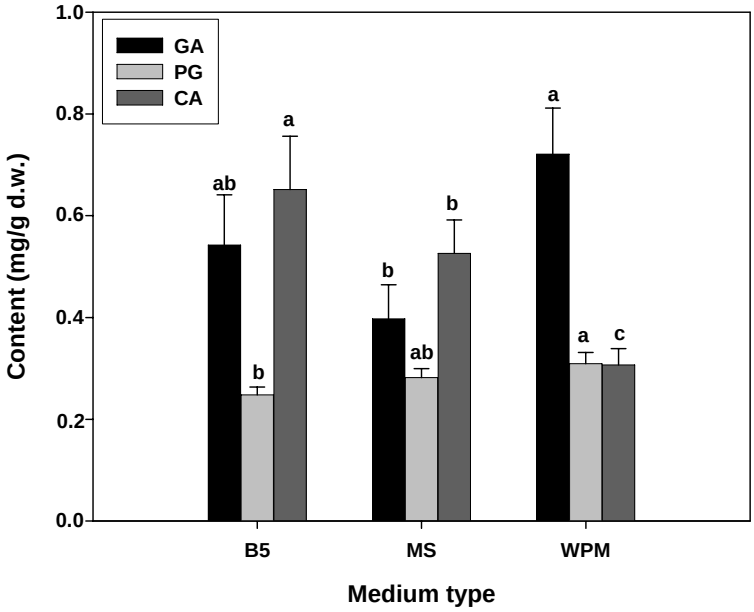


Fig 3.4.3.1.1.1.The effect of medium type on the content of GA, PG and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated ten times (n=10). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

3.4.3.1.2. Light Condition

Light conditions did not influence GA production significantly in terms of content and yield (Fig 3.4.3.1.2.1-2). Hairy roots cultured in the dark seemed to produce more GA although with no statistical significance. For PG and CA production, light conditions showed completely opposite effects. As illustrated in Figure 3.4.3.1.2.2, when exposed to light, PG production decreased, while CA yield increased. These results indicated that the metabolic pathway of CA was positively relevant to light while GA and PG metabolic pathways were

positively associated, at least partially, with the dark condition, which suggested that they played different biological roles in plant metabolism.

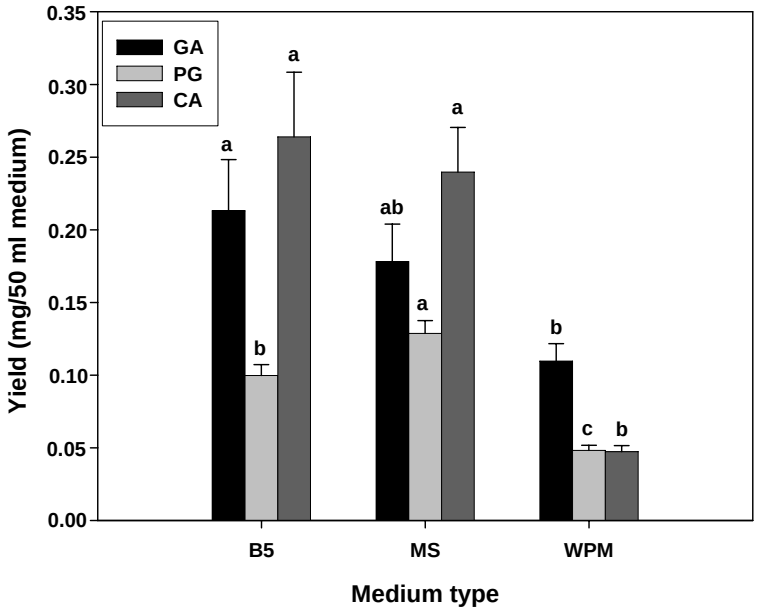


Fig 3.4.3.1.1.2. The effect of medium type on the yield of GA, PG and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated ten times (n=10). Significant differences between treatments are labeled by different letters (α = 0.05).

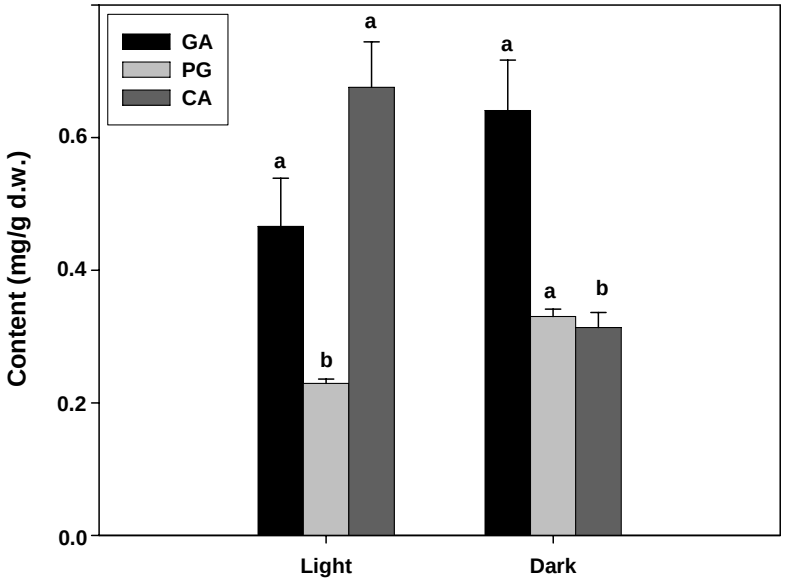


Fig 3.4.3.1.2.1. The effect of light condition on the content of GA, PG and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated fifteen times (n=15). Significant differences between treatments are labeled by different letters (α = 0.05).

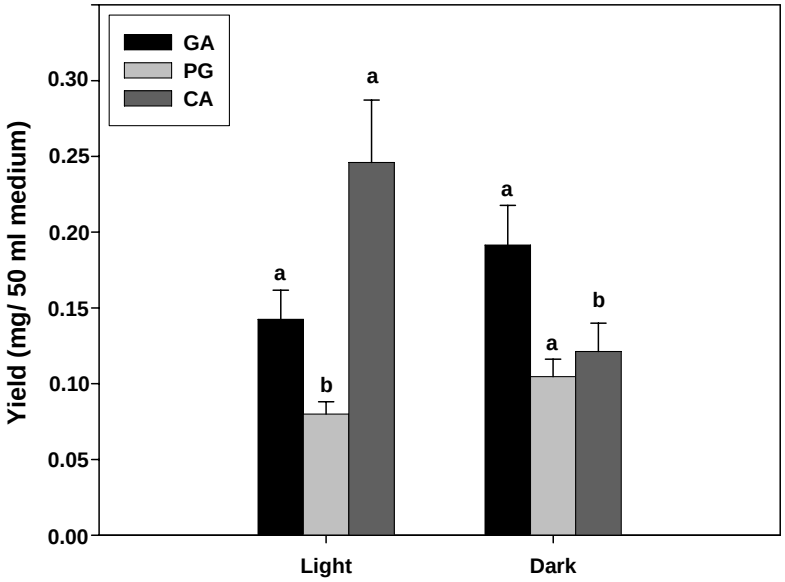


Fig 3.4.3.1.1.2. The effect of light condition on the yield of GA, PG and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated fifteen times (n=15). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

3.4.3.1.3. Initial pH

Initial pH of medium did not affect the accumulation of the three compounds too much (Fig 3.4.3.1.3.1-2). Although at pH 5.2 and 6.4 the content of PG in hairy roots was significantly different from other pH points, the yield at both pH points did not differ remarkably from other initial pH points. These results indicated a strong ability of hairy roots to adapt to or to adjust various pH of environment.

3.4.3.1.4. Auxin

Auxin type and concentration influenced both content and yield of the three compounds in hairy roots (Fig 3.4.3.1.4.1-2). Hairy roots grown without auxin showed the strongest GA synthesis ability. When auxin was added, GA synthesis dropped down. The three auxins, IBA, NAA, and 2,4-D, displayed similar inhibition pattern. As the concentrations of each auxin increased from 1μM to 10 μM, the synthesis of GA decreased accordingly. Moreover, 2,4-D, at concentration of 10 μM, suppressed GA synthesis completely. IBA showed the weakest inhibitory ability, followed by NAA.

Only IBA at concentration of 1μM seemed to enhance the content and yield of PG, but it did not significantly differ from the control. When other types of auxins or other concentrations were employed, PG synthesis was inhibited to different extents. The inhibition pattern of auxins on PG production was similar to that on GA production. In other words, the higher the concentration of auxin, the less amount of PG was produced.

IBA at concentration of 1μM could fortify CA synthesis remarkably. When IBA concentration was augmented, i.e. from 1μM to 10 μM, both the content and yield of CA tended to increase but were not significantly different from the control. NAA at concentration of 1μM barely affected CA production. But when NAA concentration increased to 10 μM, the yield of CA was strongly inhibited. 2,4-D only exhibited strong inhibitory effects on CA production at both concentrations.

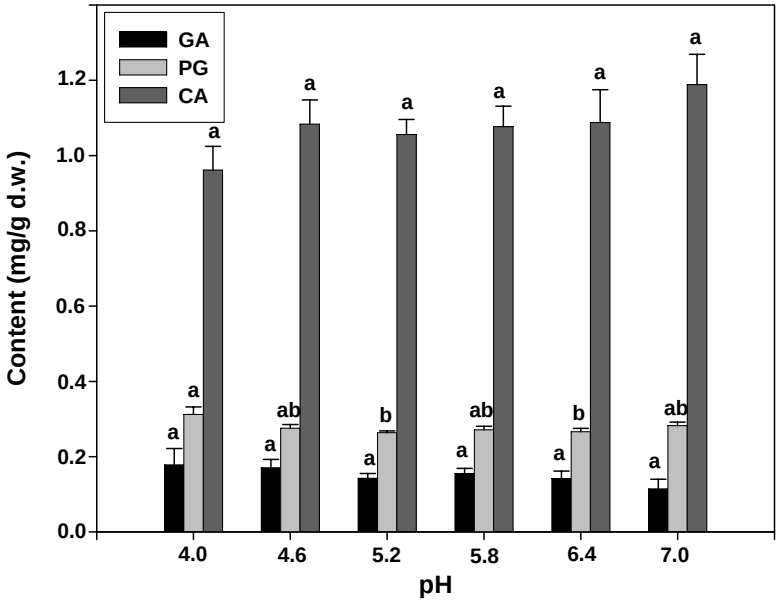


Fig 3.4.3.1.3.1. The effect of pH on the content of GA, PG and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated five times (n=5). Significant differences between treatments are labeled by different letters (α = 0.05).

3.4.3.1.5. Medium Strength

Medium strength did not affect GA content of hairy roots remarkably (Fig 3.4.3.1.5.1). But it had a significant impact on GA yield (Fig 3.4.3.1.5.2). As medium strength increased

from 0.25 to 1.0, the production of GA accelerated markedly. The increasing trend was preserved when medium strength was increased from 1.0 to 1.5, but not significantly. The impact patterns of medium strength on PG and CA production, either content or yield, were similar. Both of them showed increasing trend. But for PG production, the trend stopped when medium strength increased to 1.0. As medium strength continued to increase to 1.5, the PG production of hairy root tended to drop, although not significantly. However, the CA production continued to increase remarkably until medium strength attained 1.5.

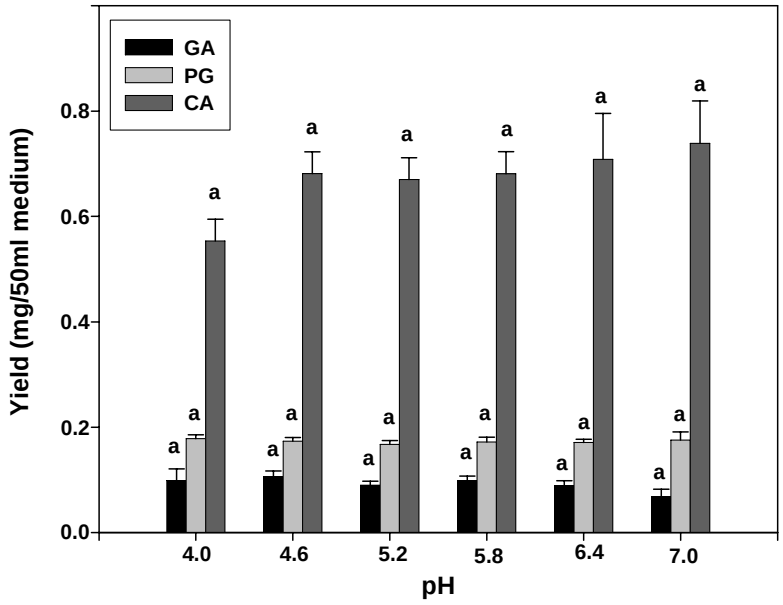


Fig 3.4.3.1.3.2. The effect of pH on the yield of GA, PG and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated five times (n=5). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

3.4.3.1.6. Sucrose Concentration

Sucrose concentration in the medium did not affect GA content significantly (Fig 3.4.3.1.6.1). However, it displayed remarkable effects on both PG and CA synthesis in hairy roots (Fig 3.4.3.1.6.2). Furthermore, the impact patterns of sucrose concentration on the two compounds' synthesis were different. As sucrose concentration increased from 1.5% to 4.5%, PG content in hairy roots decreased significantly while CA content increased markedly. As far as yields were concerned, the impact pattern of sucrose concentration on the three

compounds exhibited great similarity. In other words, when more sucrose was added into medium, more compounds were synthesized. Moreover, significant differences were observed among the three sucrose concentrations tested on all the three compounds.

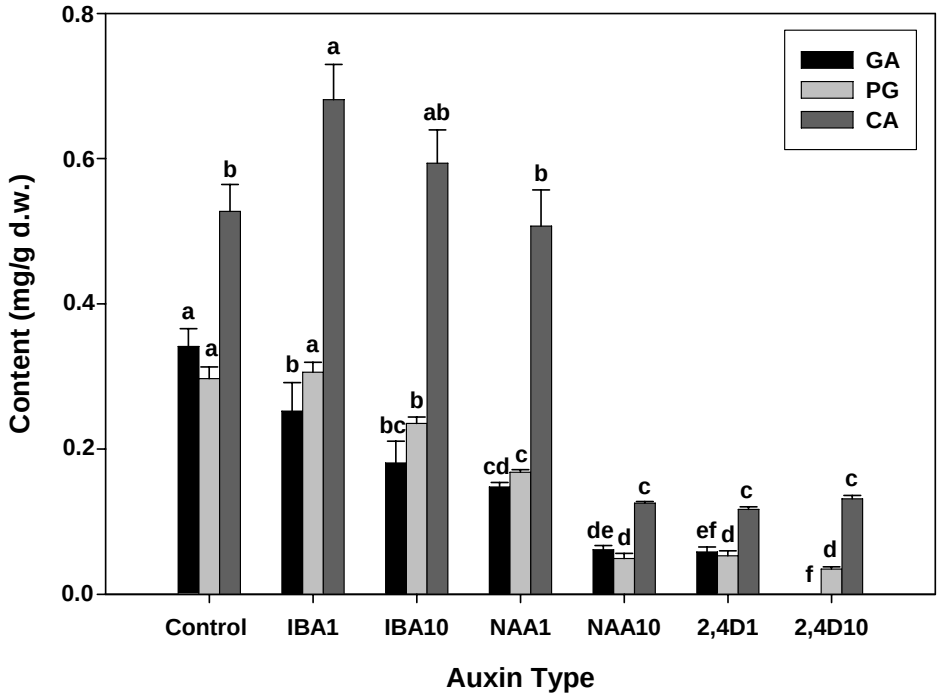


Fig 3.4.3.1.4.1. The effect of auxin type on the content of GA, PG and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated four times (n=4). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

3.4.3.1.7. Culture Volume

The volume of flasks used for hairy root culture did not influence GA and PG synthesis remarkably (Fig 3.4.3.1.7) when the volume ratio of medium and flask was kept stable (1:2.5). Although the highest content of GA and PG was observed in 250 ml and 1000 ml flasks respectively, no significant difference was observed among the four sized flasks regarding GA and PG content. But the two compounds exhibited different accumulation pattern in terms of flask volume. As culture volume increased from 125 ml to 1000 ml, GA content of hairy roots increased at first, followed by a decrease. But for PG content, it declined first and then increased gradually. However, CA content patterns in terms of culture volume were different from the other two. As culture volume increased, CA content reduced

gradually. And significant difference was observed between 125 ml and the other three sizes of flasks.

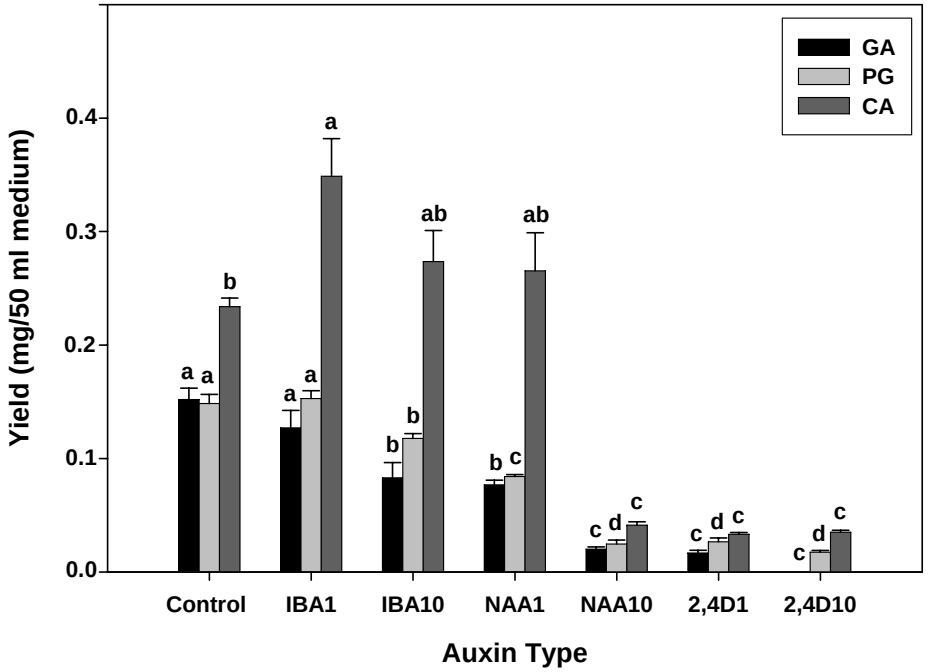


Fig 3.4.3.1.4.2. The effect of auxin type on the yield of GA, PG and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated four times (n=4). Significant differences between treatments are labeled by different letters (α = 0.05).

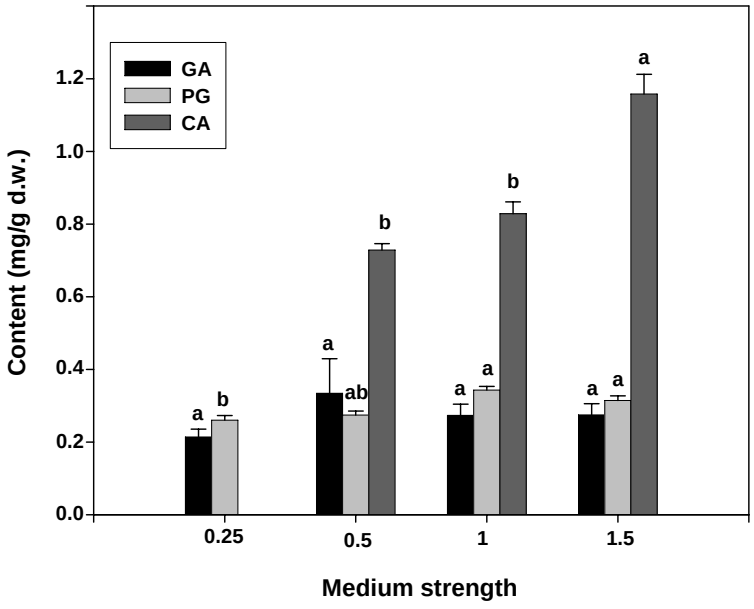


Fig 3.4.3.1.5.1. The effect of medium strength on the content of GA, PG and CA of *E. ulmoides* hairy roots. Missing bar at medium strength of 0.25 is due to shortage of samples for the analysis. Vertical bars represent standard error. Each treatment is replicated five times (n=5). Significant differences between treatments are labeled by different letters (α = 0.05).

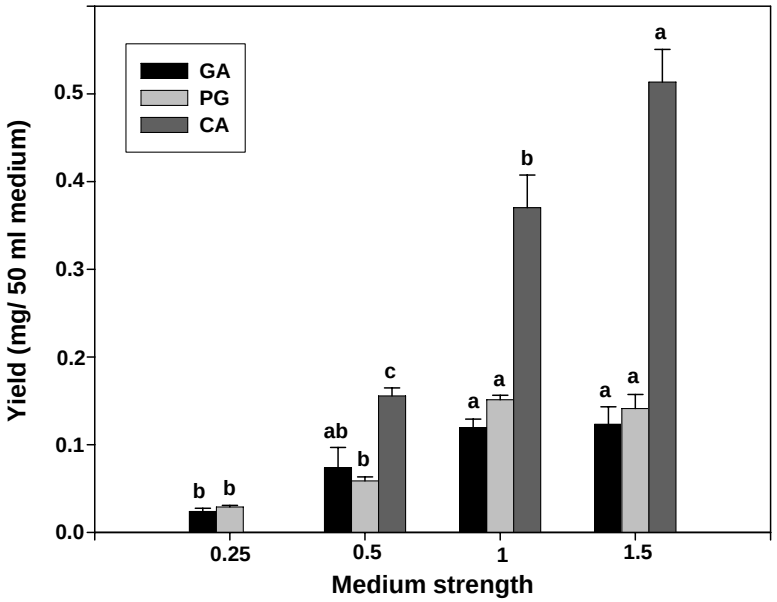


Fig 3.4.3.1.5.2. The effect of medium strength on the yield of GA, PG and CA of *E. ulmoides* hairy roots. Missing bar at medium strength of 0.25 is due to shortage of samples for the analysis. Vertical bars represent standard error. Each treatment is replicated five times (n=5). Significant differences between treatments are labeled by different letters (α = 0.05).

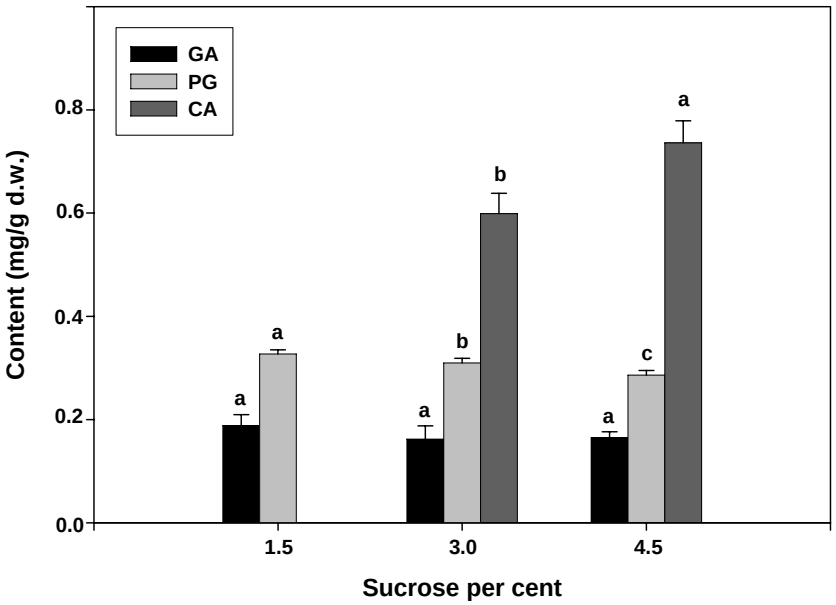


Fig 3.4.3.1.6.1. The effect of sucrose concentration on the content of GA, PG and CA of *E. ulmoides* hairy roots. Missing bar at sucrose percent of 1.5% is due to shortage of samples for the analysis. Vertical bars represent standard error. Each treatment is replicated twelve times (n=12). Significant differences between treatments are labeled by different letters (α = 0.05).

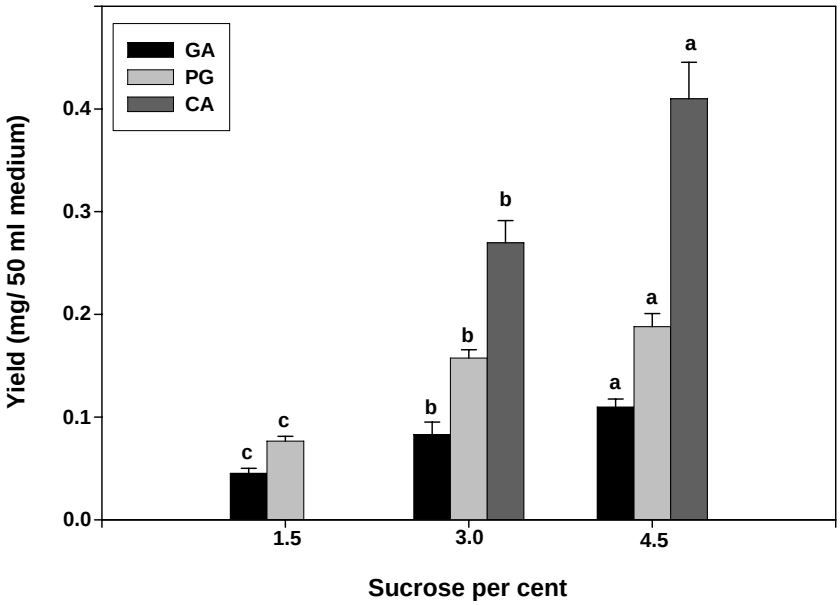


Fig 3.4.3.1.6.2. The effect of sucrose concentration on the yield of GA, PG and CA of *E. ulmoides* hairy roots. Missing bar at sucrose percent of 1.5% is due to shortage of samples for the analysis. Vertical bars represent standard error. Each treatment is replicated twelve times (n=12). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

3.4.3.1.8. Hairy Root Lines

Significant differences were observed in different hairy root lines in both content and yield of secondary metabolites (Fig 3.4.3.1.8.1-2). HRL-1 had the highest GA content, around 0.32 mg/g d.w., but did not differ much from other lines except HRL-8. HRL-1 also had the highest PG content. However, the highest CA content was observed in line HRL-8, not HRL-1. When growth factors were taken into consideration, HRL-2 was the best line for further study since it could produce the highest amount of GA and PG. For CA yield, there was no significant difference among the eight hairy root lines.

3.4.3.1.9. Interaction between Sucrose Concentration and Auxin Type

Significant interaction was found between sucrose concentration and auxin type on GA yield (Fig 3.4.3.1.9). In the medium with IBA, GA yield of hairy roots with 4.5% sucrose was significantly different from that with 3.0% sucrose as well as the control. But in the medium with NAA added, no remarkable difference in GA yield was found between 3.0% and 4.5% sucrose.

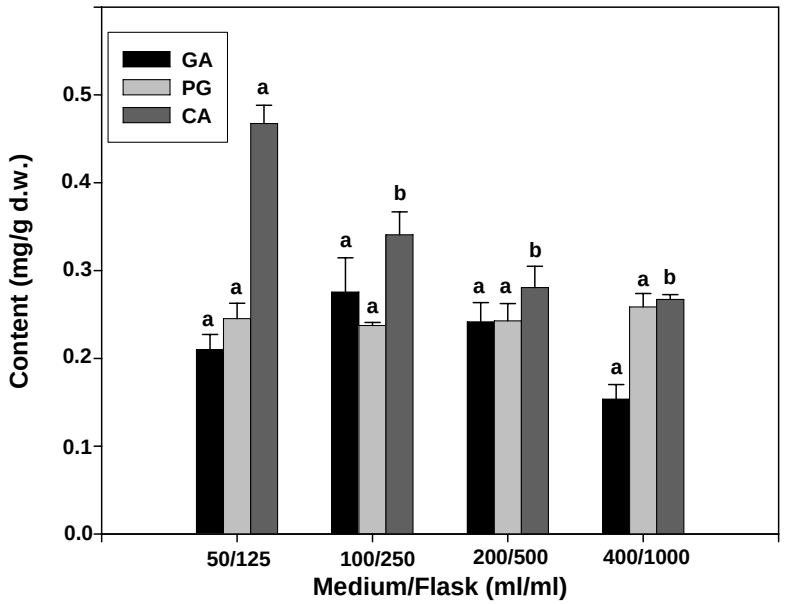


Fig 3.4.3.1.7. The effect of culture volume on the content of GA, PG and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated three times (n=3). Significant differences between treatments are labeled by different letters (α = 0.05).

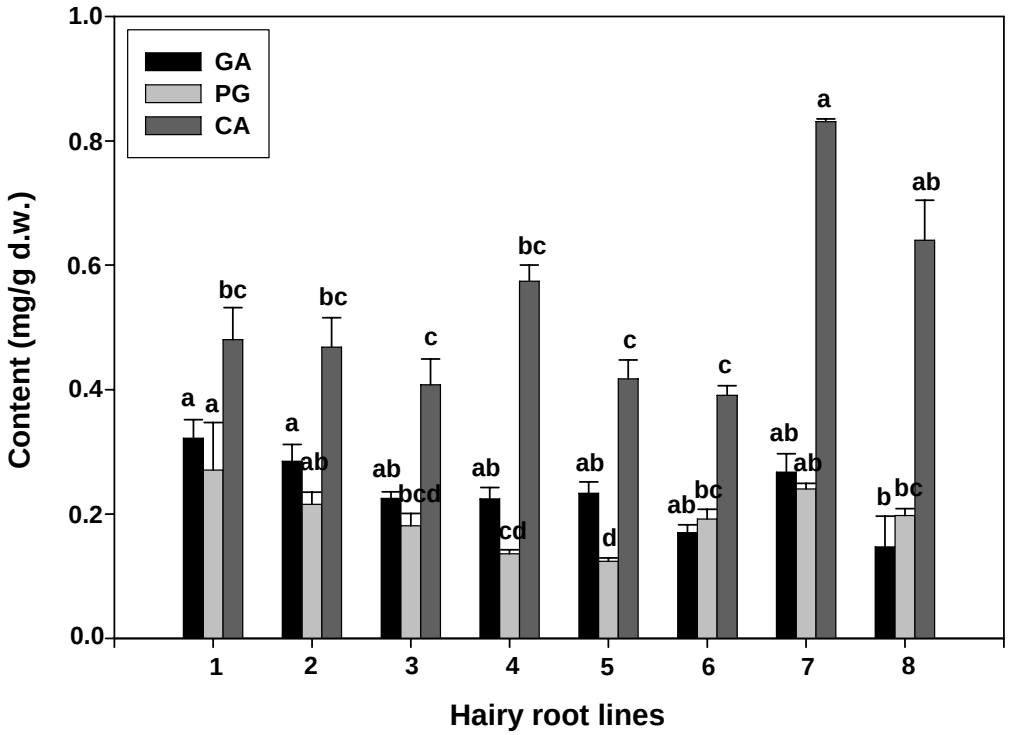


Fig 3.4.3.1.8.1. Variation of the content of GA, PG, and CA in different *E. ulmoides* hairy root lines. Vertical bars represent standard error. Each treatment is replicated three times (n=3). Significant differences between treatments are labeled by different letters (α = 0.05).

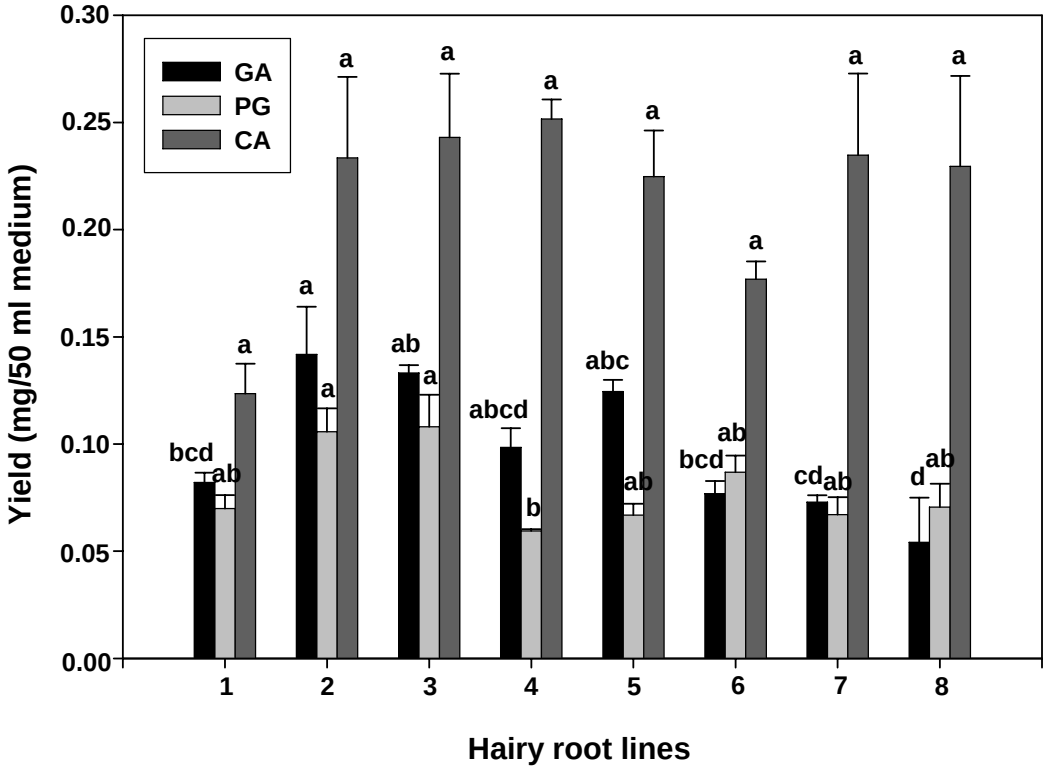


Fig 3.4.3.1.8.2. Variation of the yield of GA, PG, and CA in different *E. ulmoides* hairy root lines. Vertical bars represent standard error. Each treatment is replicated three times (n=3). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

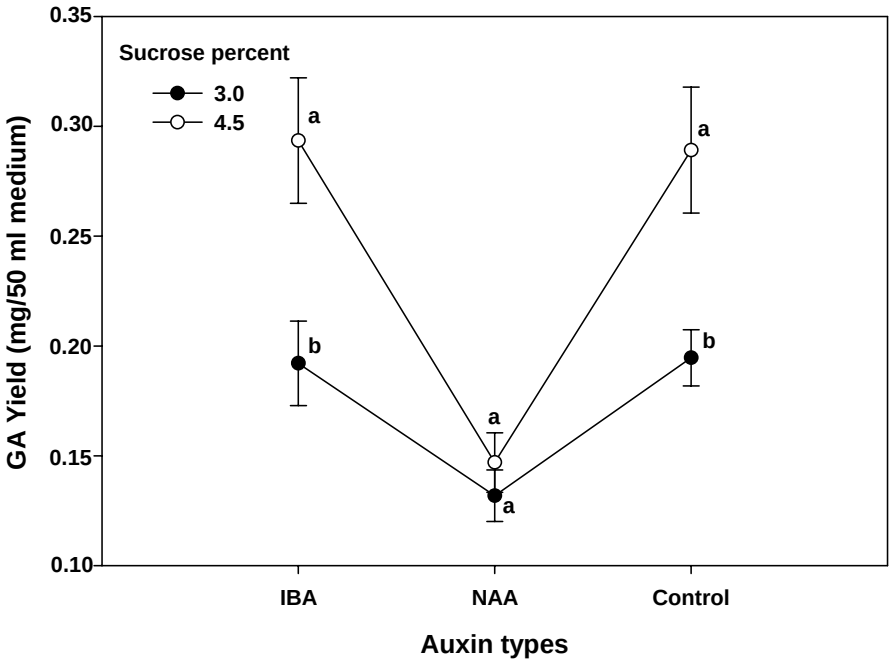


Fig 3.4.3.1.9. Interaction between auxin type and sucrose concentration on GA yield of *E. ulmoides* hairy roots. Different letters at each auxin type indicate significant difference.

3.4.3.1.10. Interaction between Medium Strength and Sucrose Concentration

Significant interaction was also found between medium strength and sucrose concentration on PG yield (Fig 3.4.3.1.10). When basal medium strength was 0.5, PG yield of hairy roots in the medium supplemented with 3.0% and 4.5% sucrose differed markedly from that with only 1.5% sucrose, but did not differ from each other. Similar phenomena occurred when basal medium strength was 1.5. However, when basal medium strength was 1.0, PG yield of hairy roots in the medium with 4.5% sucrose was higher than that with 1.5% and 3.0% sucrose significantly. Furthermore, PG yield with 3.0% sucrose was significantly different from that with only 1.5% sucrose.

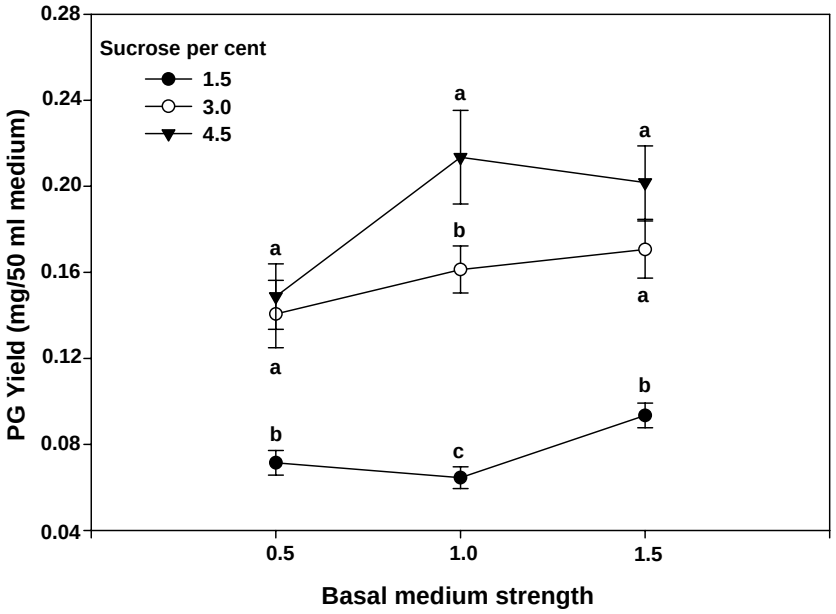


Fig 3.4.3.1.10. Interaction between basal medium strength and sucrose concentration on PG yield of *E. ulmoides* hairy roots. Different letters at each medium strength indicate significant difference.

3.4.3.1.11. Chemical Component Changes during Growth Period

Chemical analysis showed that PG content was stable, around 0.32 mg/g d.w., during the whole growth period, while GA content doubled in two weeks, from 0.36 mg/g d.w. to 0.71 mg/g d.w., and then stabilized in the following weeks (Fig 3.4.3.1.11.1). But for CA production, the content of CA kept increasing during the growth period and accumulated

almost 2.6 times in six weeks, i.e. changing from 0.83 mg/g d.w. to 2.14 mg/g d.w. The yields of PG and GA continued to increase in the first five weeks and then attained at stable level, i.e. 0.69 mg and 0.28 mg per flask, respectively (Fig 3.4.3.1.11.2). However, CA production still did not reach a stable level after six weeks and kept increasing. Compared with that in bark or leaves of field-grown trees, in which GA and PG contents were about 0.37%~3.98% and 0%~0.55% (Sha et al. 1986; Tang et al. 2004), GA and PG contents in *E. ulmoides* hairy roots were relatively lower. However, as bioactive components in *E. ulmoides* are often affected by the source from different organs, developing stages, seasons, districts, and past-harvest treatments (Gao et al. 1997; Ma 2003; Qi et al. 2003; Zhang 2002), *E. ulmoides* hairy roots could synthesize those compounds stably and were easy to culture, which indicated a prospective application.

3.4.3.2. Elicitation

3.4.3.2.1. Elicitation with MeJA

MeJA tended to inhibit GA synthesis in *E. ulmoides* hairy roots although the effect was not significant (Fig 3.4.3.2.1). As MeJA concentration increased from 0 to 50 μM, GA content in hairy roots declined from 0.863 mg/g d.w. to 0.554 mg/g d.w. But when MeJA concentration kept increasing to 100μM, GA content augmented a little and attained 0.672 mg/g d.w. The effect of MeJA on PG synthesis was totally different from that on GA synthesis. In other words, when MeJA concentration increased from 0 μM to 50 μM, PG synthesis increased too. But as MeJA increased to 100 μM, PG synthesis dropped down to 0.228 mg/g d.w. and significantly differed from other treatments. CA synthesis in *E. ulmoides* hairy roots was affected by MeJA treatments remarkably. The more MeJA added to medium, the more CA synthesized. Compared with control, 100 μM MeJA elicited 83% more CA production in hairy roots.

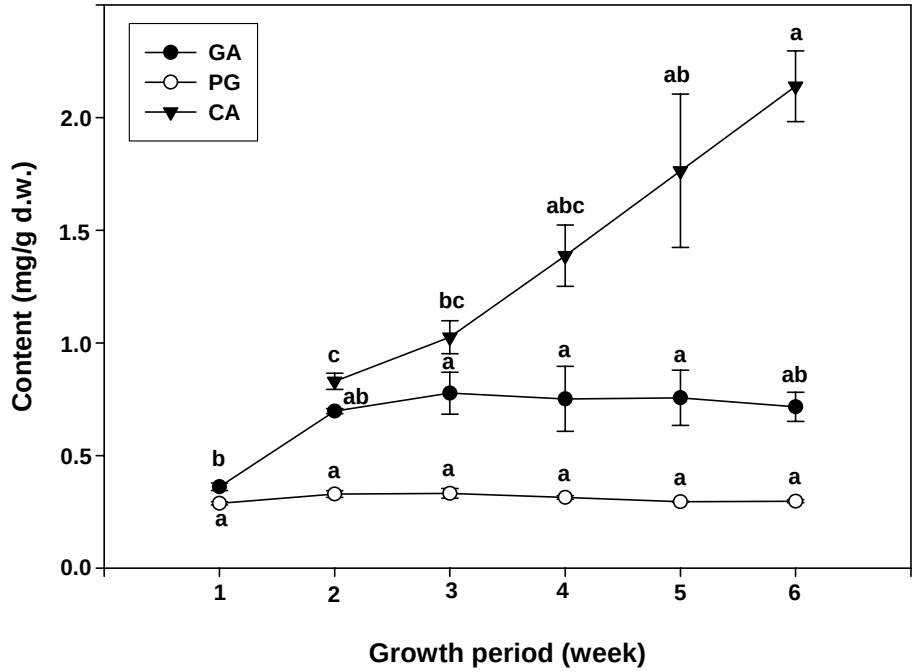


Fig 3.4.3.1.11.1. The changes of contents of GA, PG and CA during growth period of *E.ulmoides* hairy roots. Missing point at week of one is due to shortage of material for the analysis. Vertical bars represent standard error. Each treatment is replicated four times (n=4). Significant differences between treatments are labeled by different letters (α = 0.05).

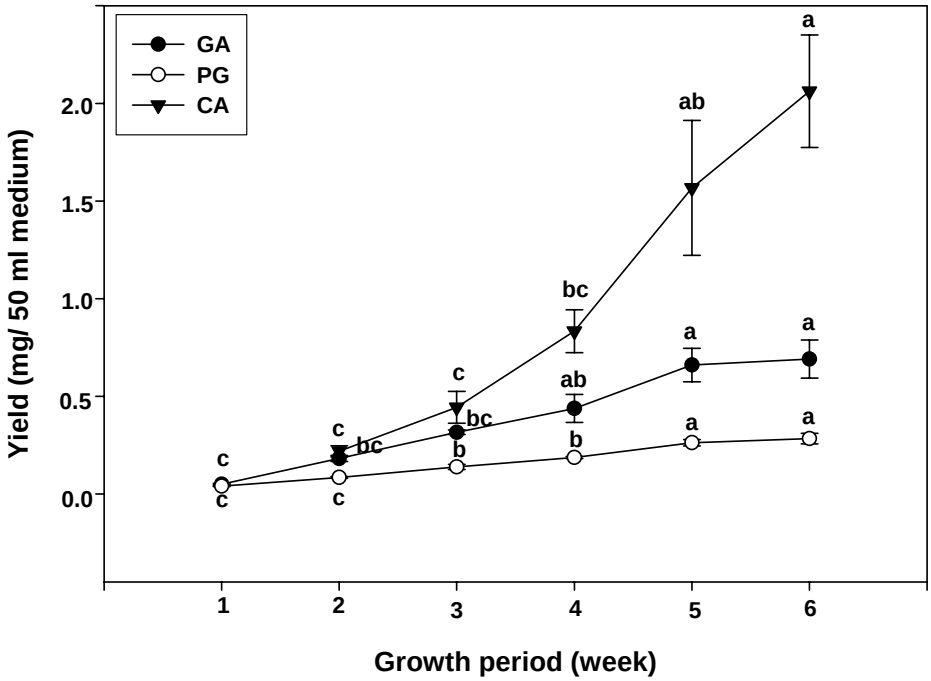


Fig 3.4.3.1.11.2. The changes of yields of GA, PG and CA during growth period of *E. ulmoides* hairy roots. Missing point at week one is due to shortage of material for the analysis. Vertical bars represent standard error. Each treatment is replicated four times (n=4). Significant differences between treatments are labeled by different letters (α = 0.05).

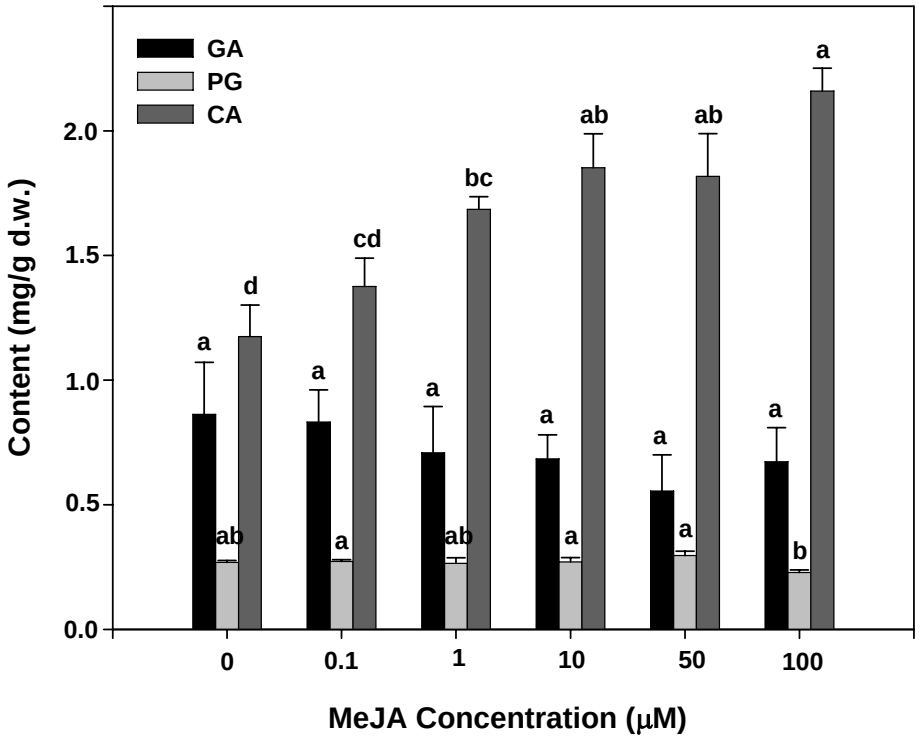


Fig 3.4.3.2.1. The effect of MeJA elicitation on the contents of GA, PG, and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated four times (n=4). Significant differences between treatments are labeled by different letters (α = 0.05).

3.4.3.2.2. Elicitation with SA

As shown in Fig 3.4.3.2.2.2, the contents of all the three compounds in hairy roots decreased in all the groups treated with different concentrations of SA when compared with the group without SA treatment. However, since SA was dissolved in 95% ethanol, another control, i.e. group treated with 100 μl 95% ethanol, should be used as a vehicle control. In contrast to the vehicle control, GA content increased up to 24% when SA was employed at concentrations below 1 mM. However, as SA concentration in the medium increased to 5 mM, GA content in hairy roots dropped dramatically to 0.032 mg/g d.w., which was only 6% of the vehicle control. A similar phenomenon was observed for PG content in hairy roots. As SA concentration increased from 0.1 mM to 1 mM, PG production continued to increasing up to 17% compared to the alternative control. But when 5mM SA was applied, PG content reduced to 0.159 mg/g d.w. For CA synthesis, the impact pattern of SA was a little different.

At low concentration, such as 0.1 mM, SA tended to accelerate CA synthesis but not significantly. When more SA was added to the medium, less CA was synthesized. As SA concentration increased to 5 mM, CA content of hairy roots was only 48% of the vehicle control. Moreover, addition of SA at high concentrations, more than 0.5 mM, changed the morphology of hairy roots and caused the exudation of secondary metabolites into medium (Fig 3.4.3.2.2.1,3).

Fig 3.4.3.2.2.1. SA elicitation on *E. ulmoides* hairy roots

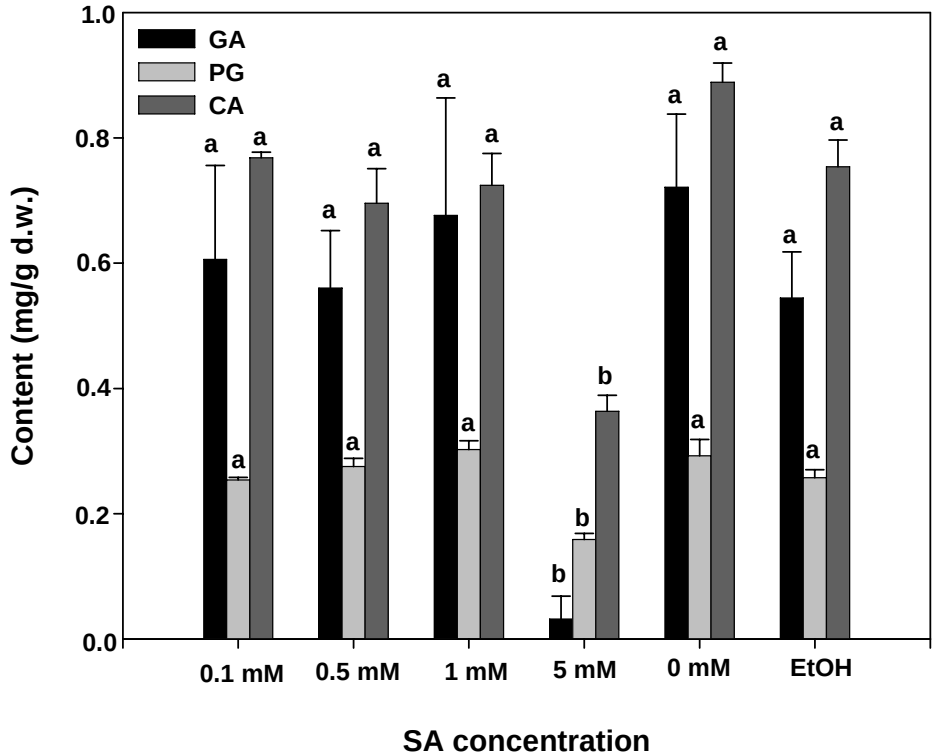


Fig 3.4.3.2.2.2 The effect of SA elicitation on the contents of GA, PG and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated four times (n=4). Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

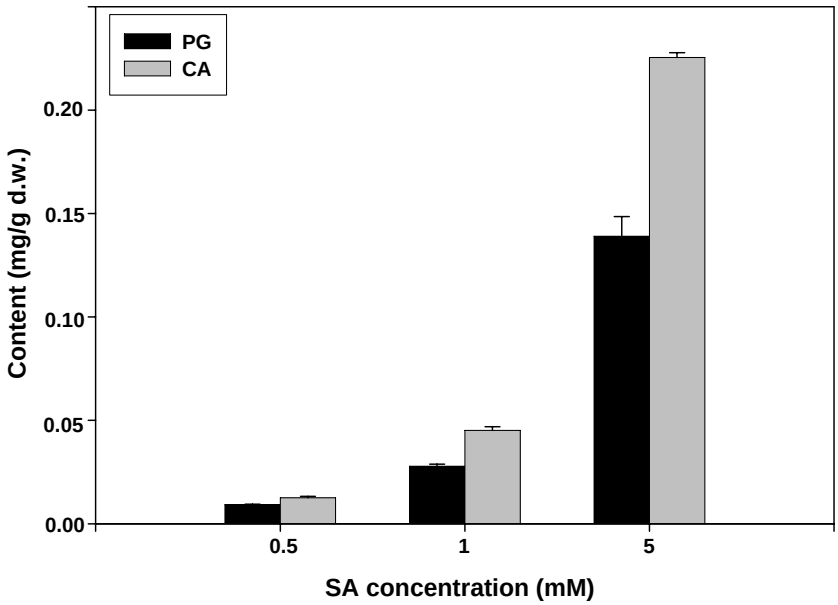


Fig 3.4.3.2.2.3 The content changes of PG and CA in culture medium elicited by SA. Vertical bars represent standard error. Each treatment is replicated four times (n=4).

3.4.3.3.3. Light Elicitation

Exposure to light seemed to have some impact on the synthesis of secondary metabolites, especially on GA and CA, of *E. ulmoides* hairy root although not significantly (Fig 3.4.3.3.3.1-2). As illustrated in Fig 3.4.3.3.3.1, GA and CA contents of hairy roots exposed to light increased 0.39 fold and 0.38 fold respectively than that cultured in the dark. Moreover, when the hairy roots cultured in the dark were exposed to light for two days, both GA and CA contents increased and attained 0.189 mg/g d.w. and 1.249 mg/g d.w. respectively, which was a 30% increase over those cultured in the dark. But for PG synthesis, exposure to light seemed to inhibit its production. As shown in Fig 3.3.4.6.3.1, the PG content of hairy roots in light conditions decreased slightly than that in dark conditions. The highest yield of all the three compounds was found under the light elicitation condition, i.e. hairy roots were cultured in dark for four weeks prior to exposure to light. Significant difference was found on CA yield in hairy roots cultured under light and in the dark, respectively.

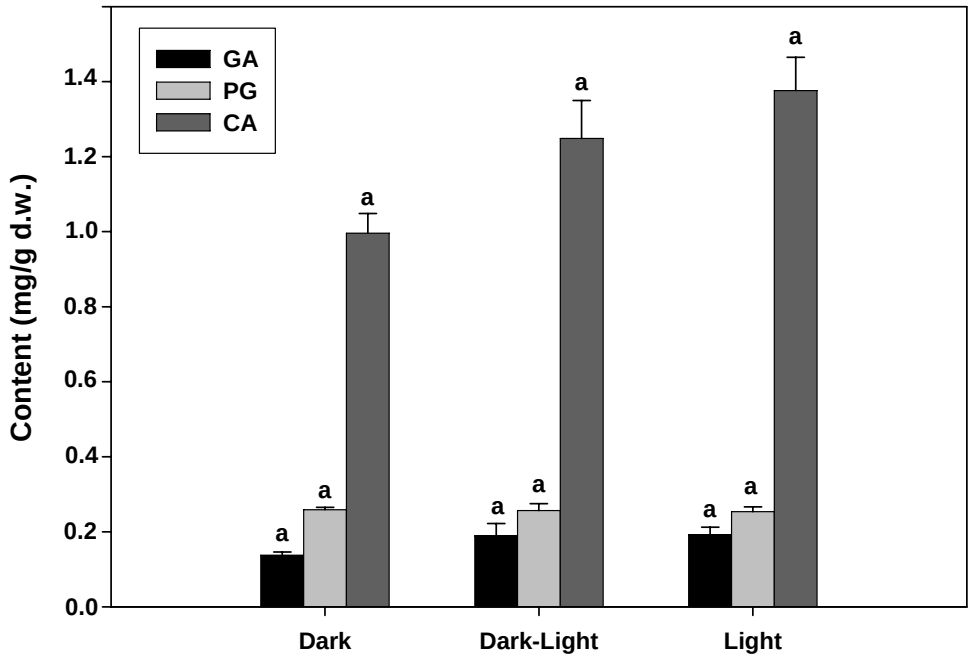


Fig 3.4.3.3.3.1. Light condition on the content of GA, PG and CA of *E. ulmoides* hairy roots. Vertical bars represent standard error. Each treatment is replicated three times (n=3). Significant differences between treatments are labeled by different letters (α = 0.05).

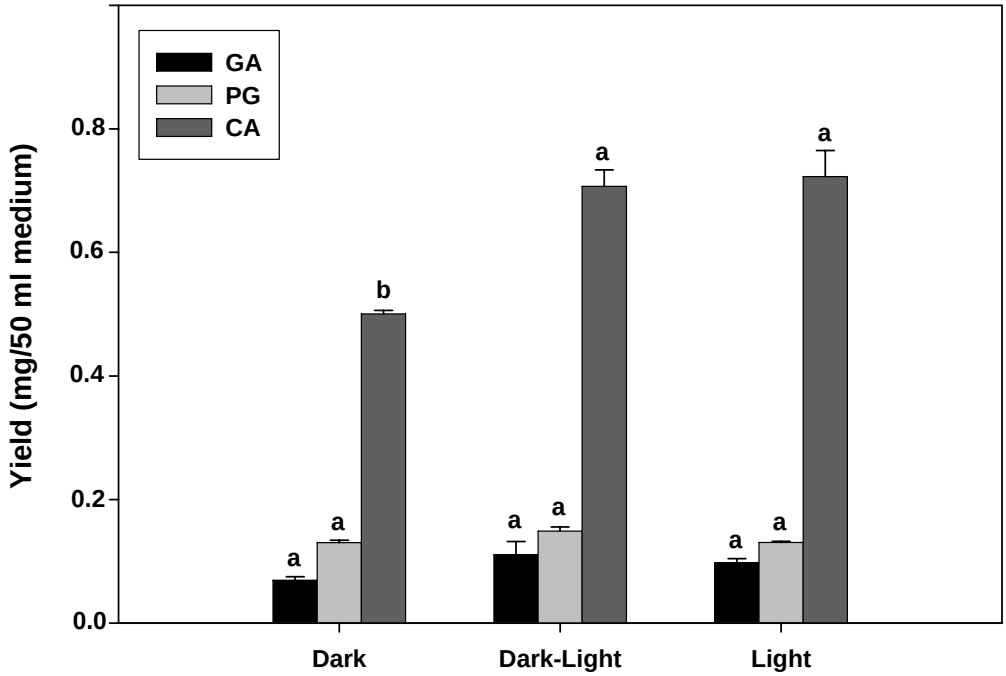


Fig 3.4.3.3.3.2. Light condition on the yield of GA, PG and CA of *E. ulmolides* hairy roots. Vertical bars represent standard error. Each treatment is replicated three times (n=3). Significant differences between treatments are labeled by different letters (α = 0.05).

3.4.3.4. Comparison of Chemical Components from Different *E. ulmoides* Sources

3.4.3.4.1. Comparison of the Contents of GA, PG and CA

Variations were found in GA, PG and CA production from different sources of *E. ulmoides* (Table 3.4.3.4.1). The highest GA production was found in root bark of *E. ulmoides*, which was 6.988 mg/g d.w. and significantly different from other sources of *E. ulmoides*. The second higher of GA production was stem bark, followed by hairy root. But no GA synthesis was detected in either callus or suspension cell cultures. Stem bark stored the highest amount of PG and significantly differed from other sources of *E. ulmoides*. Hairy roots synthesized the second highest amount of PG followed by root bark. Only trace amounts of PG was detected in callus of *E. ulmoides*. But no PG synthesis was found in suspension cell culture of *E. ulmoides*. For CA synthesis, the highest content was found in hairy roots, followed by stem bark. The amount of CA synthesized in root bark was similar to that in suspension cell and callus cultures.

Table 3.4.3.4.1. Comparison of GA, PG and CA contents from different sources of *E. ulmoides*. Significant difference are labeled in different lowercase letters.

Sample Source	GA content (mg/g d.w.)	PG content (mg/g d.w.)	CA content (mg/g d.w.)
Callus (2 months)	0	0.006 ± 0.000	0.178 ± 0.003^{c}
Suspension Cell (2 months)	0	0	0.294 ± 0.011^{c}
Hairy Root (4 weeks)	0.881 ± 0.088^{c}	0.317 ± 0.010^{b}	1.504 ± 0.098^{a}
Root Bark (6 years)	6.988 ± 0.031^{a}	0.217 ± 0.018^{c}	0.200 ± 0.007^{c}
Stem Bark (6 years)	2.122 ± 0.047^{b}	1.196 ± 0.016^{a}	1.134 ± 0.037^{b}

3.4.3.4.2. Comparison of Chemical Fingerprints

Comparison of chemical fingerprints of the extracts revealed differences in chemical composition or relative content of chemical components from different *E. ulmoides* sources (Fig 3.4.3.4.2.1-3). It is generally assumed that hairy root cultures closely resemble the roots of the plant from which they were derived with respect to their secondary metabolite synthetic capacity (Nguyen et al. 1992). However, as illustrated on Fig 3.4.3.4.2.1 to Fig

3.4.3.4.2.3, the profile of secondary metabolites in hairy roots was remarkably different from that in other tissues, including callus, suspension cell, root bark and stem bark. Fig 3.4.3.4. 2.1 and Fig 3.4.3.4.2.2 showed the fingerprints of methanol extracts from different sources of *E. ulmoides*, while Fig 3.4.3.4.2.3 displayed the fingerprints of water extracts. For the methanol extracts, many specific peaks appeared only in the fingerprints of hairy roots, for instance, the peak at 16.5 min detected at 226 nm and the peak at 19.0 min detected at 236 nm. These peaks implicated new compounds or components that might not found in stem or root barks. The water-soluble components in hairy roots were relatively simple. As shown in Fig 3.4.3.4.2.3, water extract of hairy roots shared the three main peaks with that from other sources. Root bark extract seemed to be the most complicated since many peaks were found, followed by callus extract. The sharing of three peaks suggested the important role of the compounds that they played in physiological activity.

3.5. Discussions

3.5.1. Medium Type

Many reports described the effects of medium composition on the growth rate of hairy roots. Nutritional factors might affect the number of lateral branches per unit length or the average cross-sectional dimensions of the roots (Hilton et al. 1990). MS, B5 and WPM are the three standard types of most widely used media for the culture of hairy roots of many species. It was inferred that the difference in their ionic strength might be the primary factor affecting the growth rates of hairy roots.

As demonstrated by Saenz-Carbonell et al (1997), hairy roots are less susceptible to manipulation by changes in medium composition than callus and cell suspension cultures. However, when the individual components of the medium were modified one by one, several of them modified the yield of root cultures. Nitrogen (N) source changes, the absence of phosphorus (P), calcium (Ca), and cobalt (Co), and the increase in the sucrose content of the

medium increased the level of alkaloids in *Datura stramonium* hairy root cultures. The use of ammonium as a N source provoked alkaloid excretion into the medium. For *Psoralea* species, nitrate concentrations ranging from 30 to 60 mM did not affect hairy root growth (Nguyen et al. 1992). However, either higher concentration (100 mM) or lower concentration (5 mM) inhibited the growth of hairy roots. When we compared the three media, MS, B5 and WPM, the contents of phosphorus and calcium were similar, while the content of nitrogen was different. MS and B5 contained more nitrogen, approximately 22.5 mM and 26.8 mM, than that in WPM, about 9.7 mM. Therefore, the content of nitrogen might be an important factor that affected the growth of *E. ulmoides* hairy roots.

However, studies also showed that the highest content of GA and PG was found in *E. ulmoides* hairy roots grown in WPM medium, which indicated that high concentration of nitrogen might inhibit GA and PG synthesis. For CA synthesis, the situation was completely reversed. The lowest content of CA was achieved in hairy roots cultured in WPM medium, which suggested the possible enhancing effect of high concentration of nitrogen in CA synthesis. Further studies should be undertaken to prove the hypothesis.

3.5.2. Light

When hairy roots were exposed to light, some of them would turn green but kept similar ability to synthesize metabolites as that cultured in dark (Giri et al. 2001). But no green hairy roots of *E. ulmoides* were observed in our experiments. Light condition affected the growth rate of *E. ulmoides* hairy roots in the same way as *Datura stramonium* and *Rheum palmatum* hairy roots (Chang et al. 1998; Maldonado-Mendoza et al. 1995) but differed from *Paulownia tomentosa* hairy roots, which displayed slightly improved final biomass yields (Wysokinska et al. 1998).

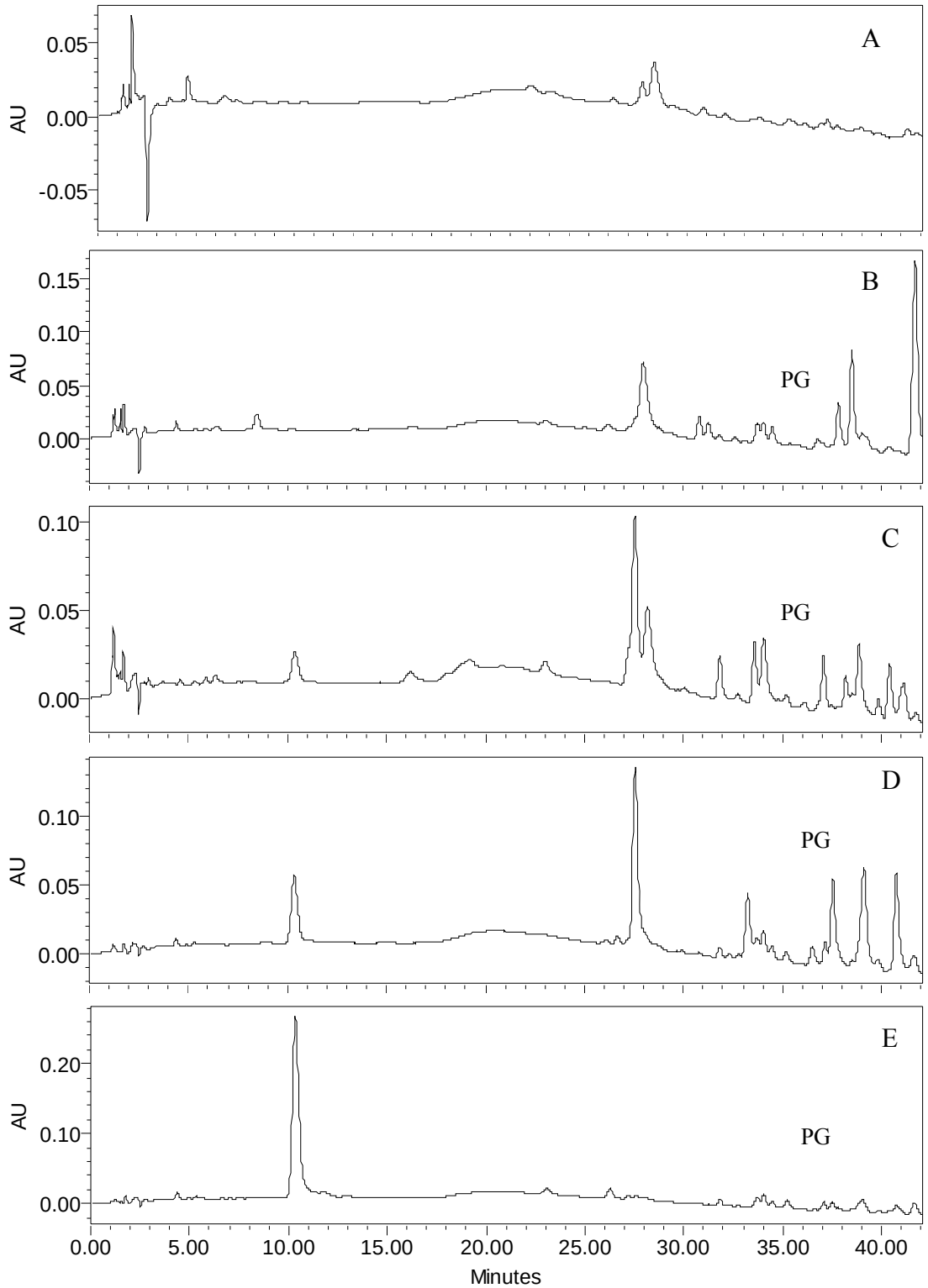


Fig 3.4.3.4.2.1. Comparison of chemical fingerprints of *E. ulmoides* from different sources detected at 226 nm. A: Suspension cells; B: Callus; C: Hairy roots; D: Stem bark; E: Root bark.

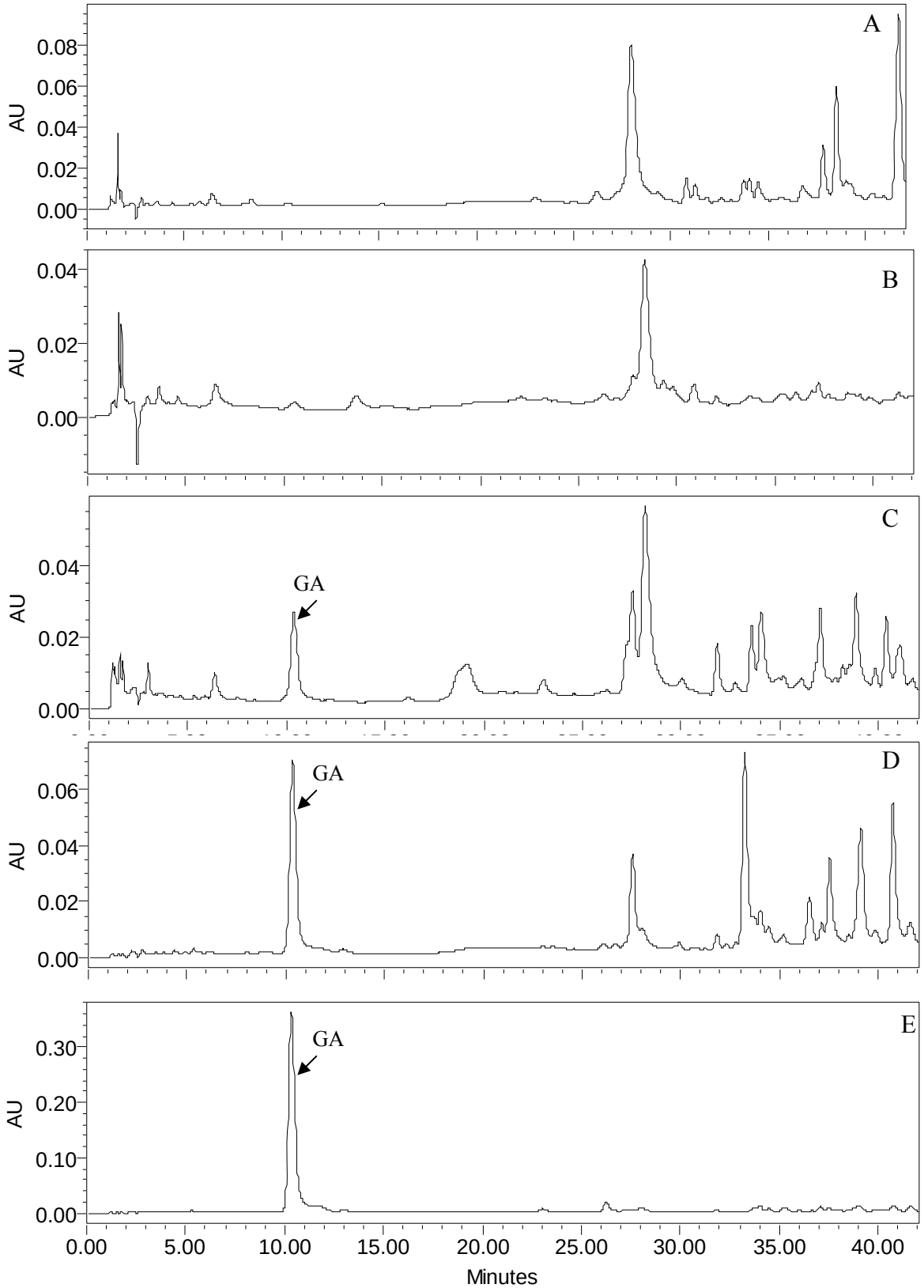


Fig 3.4.3.4.2.2. Comparison of chemical fingerprints of *E. ulmoides* from different sources detected at 236 nm. A: Suspension cells; B: Callus; C: Hairy roots; D: Stem bark; E: Root bark.

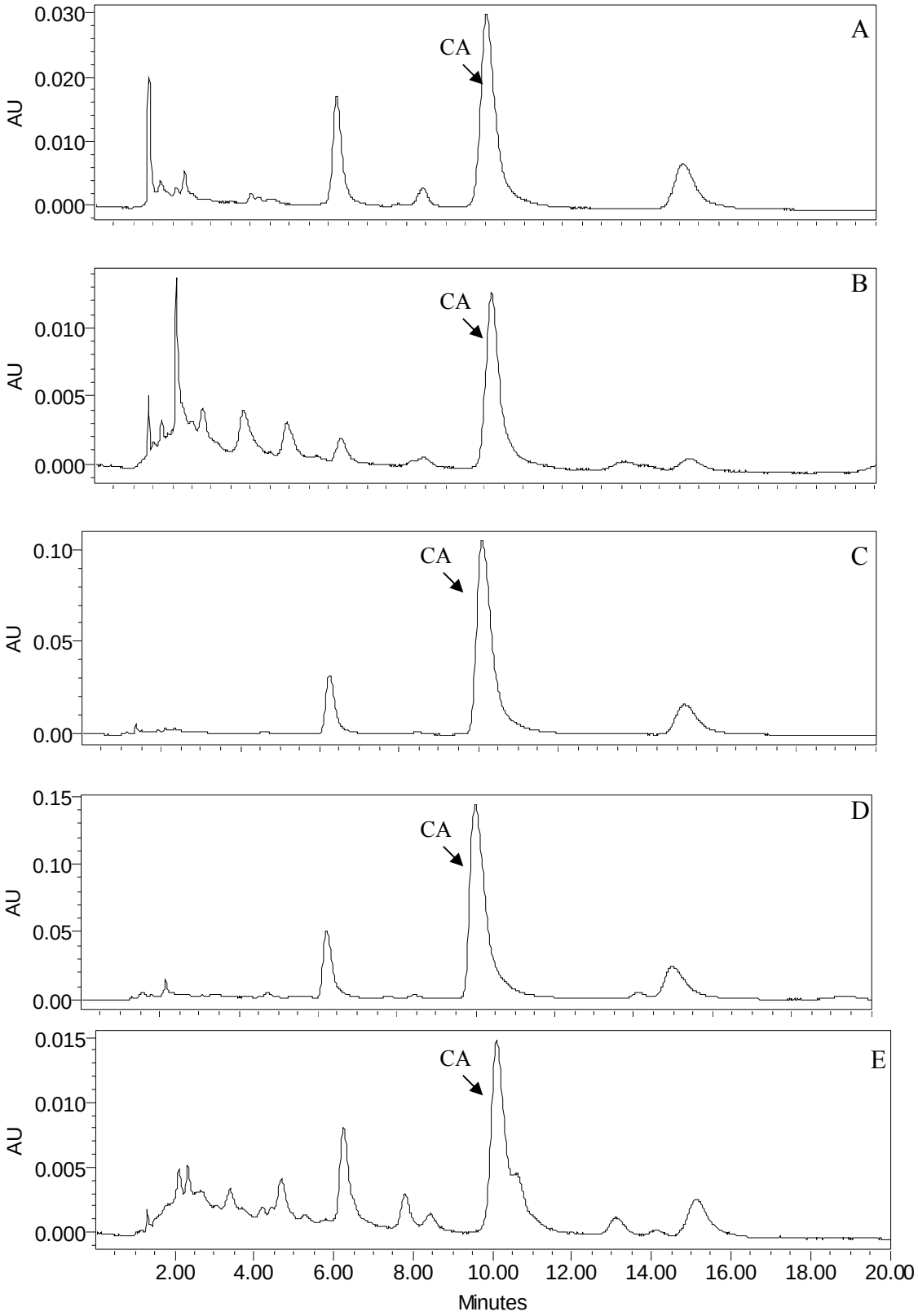


Fig 3.4.3.4.2.3. Comparison of chemical fingerprints of *E. ulmoides* from different sources detected at 325 nm. A: Suspension cells; B: Callus; C: Hairy roots; D: Stem bark; E: Root bark.

3.5.3. Medium Strength

For some species, the medium strength seemed not to influence the growth of hairy roots. As reported by Giri et al (2001), hairy roots of *Artemisia annua* grew well on 1.0, 0.5 and 0.25 strength MS media. But for *P. tomentosa*, the hairy roots did not grow in full strength B5 and MS media (Wysokinska et al. 1998). When medum was diluted to half strength, either B5 or MS, they grew rapidly. On the other hand, hairy roots of *P. tomentosa* cultured in full strength WPM medium showed 2-times higher yield of biomass than those cultured in 0.5 WPM medium. For *Solanum mauritianum* hairy roots, there was no obvious difference in the appearance grown on half strength and full strength medium (Drewes et al. 1995). Our observations showed that full strength MS medium was the best for the growth of *E. ulmoides* hairy roots, while other strengths inhibited the growth.

Different impact patterns of the changes of medium strength on the synthetic secondary metabolites were found in different species. As the medium strength decreased to half strength, solasodine level in *Solanum mauritianum* hairy roots was lowered but ajmaline and ajmalicine levels in *Pauvolfia micrantha* were increased (Drewes et al. 1995; Sudha et al. 2003). Synthesis of secondary metabolites in *E. ulmoides* hairy roots showed similar responses to the changes of medium strength as *S. mauritianum* hairy roots, i.e. synthesis was reduced according to the lowering of medium strength.

3.5.4 Initial pH

The experimental results showed that MS medium with pH 5.5 to 5.8 is suitable for growth of the *Rheum palmatum* hairy roots (Chang et al. 1998).Our studies showed that MS medium with pH 6.4 was the most suitable condition for growth of the *E. ulmoides* hairy roots but was not significantly different from pH 5.8. Nevertheless, the highest contents of GA and PG were found in hairy roots cultured in medium with pH 4.0, while the highest content of CA was in medium with pH 7.0. The results indicated that acidic initial condition

might favor GA and PG synthesis while basic initial condition tended to favor CA synthesis. But in general, no significant difference on secondary metabolite production was found among the six initial pHs.

3.5.5 Sucrose Concentration

For hairy roots of *Psoralea* species, the growth was dramatically decreased in media containing concentrations of sucrose either above or below the normal level (3%) (Nguyen et al.1992). Furthermore, high concentrations strongly inhibited growth of *Psoralea* hairy roots and modified the root morphology; roots callused and lateral branching was inhibited, probably due to osmotic stress. However, *E. ulmoides* hairy roots grew better in high concentrations of sucrose.

The maximal biomass accumulation of *E. ulmoides* hairy roots was achieved with 4.5% sucrose, whereas the maximum PG content was obtained with 1.5% sucrose. Similar results had also been achieved in hyoscyamine accumulation of *H. muticus* hairy roots (Wilhelmson et al. 2006). A high sucrose concentration might lead to high glycolysis and respiration rates that accelerated biomass production thus overriding PG production. However, the impact of sucrose concentration on CA content was quite different as that on PG content. CA synthesis was accelerated significantly with increasing sucrose concentration. This result strongly suggested an important role for CA in growth of *E. ulmoides* hairy roots. For GA production, the effect of sucrose concentration was not so remarkable, which indicated CA metabolism might not be strongly associated with carbohydrate metabolism.

3.5.6. Auxin

It was reported that exogenous IAA had scarcely any effect on cell growth, while the other three artificial auxins, IBA, NAA and 2,4-D, could increase the growth of the hairy roots(Deno et al, 1987). But the response of hairy roots to different auxins varied. In hairy roots of Pueraria lobata, lateral root formation was depressed by NAA and promoted by IBA

(Liu et al. 2002). 2.5 μM IBA promoted the best growth for hairy roots of Panax by accelerating the lateral root formation (Washida et al. 2004). IAA was observed to activate the growth of Rheum palmatum hairy roots but did not affect the growth of Hyoscyamus muticus roots (Change et al. 1998; Vanhala et al. 1998). Also NAA showed no effect on the growth of H. muticus hairy roots. Our observations revealed that lower concentration of these auxins, such as 1μM IBA and NAA, could accelerate the growth of E. ulmoides hairy root, but not higher concentrations, i.e. 10μM. Furthermore, 2,4-D inhibited the growth in both low and high concentrations. The different responses of hairy roots to auxins might be caused by different environmental selection pressures as the different species evolved to accommodate their environments.

Correspondingly, secondary metabolism was also influenced by auxin type and concentration. IBA promoted the growth best for Panax hairy roots but the highest content of ginsenoside was obtained in hairy roots supplemented with the same concentration of NAA (Washida et al. 2004). And combination of IBA and NAA contributed to increase the yield of ginsenoside. IAA inhibited the biosynthesis of free anthraquinones from *R. palmatum* hairy roots (Chang et al. 1998). Alkaloid accumulation in *H. muticus* roots was doubled in medium supplemented with IAA and NAA (Vanhala et al. 1998). For *E. ulmoides*, GA synthesis was inhibited by the three types of auxins, IBA, NAA and 2,4-D. IBA could enhance PG and CA synthesis. NAA did not affect CA production at low concentration but inhibited its production at high concentration. NAA also showed inhibitory activity on PG synthesis. 2,4-D always displayed inhibition effect on both PG and CA synthesis no matter the concentration.

3.5.7. Elicitation

Methyl jasmonate (MeJA) and jamonic acid are natural compounds occurring throughout the plant kingdom. They were recognized as phytohormones involved in many development-

related physiological processes such as senescence and germination, tuber formation or ethylene biosynthesis at low concentrations (Rickauer et al. 1997). Studies showed that addition of MeJA caused an increased accumulation of all monitored diacetylenes in *T. parthenium* hairy roots (Stojakowska et al. 2002), and ajmalicine and catharanthine in *Catharanthus roseus* hairy roots (Vazquez-Flota et al, 1994). Our studies revealed that when used at low concentrations, ranging from 0-50 μM, MeJA could accelerate PG and CA synthesis while inhibit GA production. However, when the concentration increased to 100 μM, the impact pattern changed. PG synthesis was dropped down while GA production was activated and kept increasing as well as CA content. The results suggested the synthetic pathways of these compounds were differentially regulated in *E. ulmoides* hairy roots.

Salicylic acid (SA) is a kind of phenolic compound involved in various physiological events of plants and is produced through the phenylpropanoid pathway. For instance, SA is the signal chemical of plants in response to dramatic temperature changes, pathogen and herbivore attacks (Fukami et al. 2002). Usually, excessive SA causes toxicity. Root growth of *Vicia faba* was decreased considerably when treated with SA higher than 3.5 mM (Manthe et al. 1992). SA, in a range of concentration 0.5-1.0 mM, was highly detrimental for the root growth and changed morphology of treated feverfew roots (Stojakowaska et al.2002). After 48 hr of treatment, the roots became brownish, thick and developed less laterals. At 1.0 mM concentration, SA caused necrosis of *T. parthenium*. To avoid the toxicity of SA on the growth of *E. ulmoides* hairy roots, we added the compound into medium after four weeks growth. After 48 hr co-culture, significant morphology changes were observed in hairy roots treated with 1 mM and 5 mM SA. Furthermore, PG and CA were secreted into and caused the color changes of medium. A similar phenomenon was observed in *Brugmansia candida* hairy roots as higher SA concentration caused the release of scopolamine and hyoscyamine into culture medium (Pitta-Alvarez et al. 2000). Further chemical analysis of *E. ulmoides* hairy

roots revealed that SA used below 1 mM could slightly improve GA and PG synthesis. But CA content was only increased in hairy roots treated with 0.1 mM SA. When higher concentrations of SA were employed, CA synthesis was attenuated. As 5 mM SA was added, the synthesis of all the three compounds was reduced remarkably.

3.6. Summary

Light, initial pH, and culture volume did not affect the growth remarkably, while medium type, medium strength, sucrose concentration and auxin influenced the growth significantly. Initial pH only affected PG content, while culture volume tened to have some effect on CA content. Other factors demonstrated varied impacts on content and yield of secondary metabolites.

Zero to 50 μM MeJA elicited PG synthesis but inhibited GA production. CA synthesis was enhanced by MeJA elicitation. High concentration of SA, more than 0.1 mM, reduced CA synthesis. At low concentration, i.e. less than 1 mM, SA accelerated GA and PG synthesis slightly. When used at 5 mM, SA attenuated secondary metabolite synthesis as well as acceleration the exudation of the metabolites into medium. Light exposure increased GA and CA synthesis but inhibited PG production.

Comparison of chemical profiles showed that methanol extract from hairy roots differed from other sources in chemical composition while water extract shared the three major peaks with other sources. Chemical analysis showed that secondary metabolites synthesized in hairy roots were higher than that in callus or cell cultures but comparable to that in stem or root barks.

CHAPTER 4. ENHANCING SECONDARY METABOLITE SYNTHESIS IN HAIRY ROOTS BY THE TRANSFORMATION WITH *VITREOSCILLA* HEMOGLOBIN

4.1. Background

Hairy root culture system of *E. ulmoides* had already been established and the effects of various culture conditions, including pH, medium type, auxin, culture volume, light conditions, and elicitors, had been investigated in order to achieve as high a production of secondary metabolites as possible. Another way to increase the growth and biosynthesis of secondary metabolites of hairy roots is the use of an enhancer protein from an exogenous source through transgenic techniques. Among the enhancer proteins, *Vitreoscilla* hemoglobin (VHb) is a soluble dimeric hemoprotein found in the Gram-negative aerobic bacterium *Vitreoscilla* sp., strain CI (Dikshit et al. 1989). The protein increases in concentration when the cells are grown at low levels of oxygen. The *Vitreoscilla* globin (*vgb*) gene encoding for VHb is regulated by a special promoter that is preferentially activated in response to oxygen limitation (Joshi et al. 1994). Bacterial cells transformed with *vgb* showed increased growth rate or corresponding secondary metabolites. Shake-flask cultivations of the transformed *Saccharopolyspora erythraea* strain with integrated *vgb* in its chromosome produced approximately 60% more erythromycin than the original VHb-negative strain (Bruentker et al. 1998). Transformed *Pseudomonas aeruginosa* and *Xanthomonas maltophilia* with VHb significantly increased cell viability after extended times in culture (Liu et al. 1995). Yeast strain Saccharomyces sp. X-62 containing recombinant plasmid pVgb-EX2 exhibited improved D-arabitol productivity and yield of fermentation (He et al. 2001). Plants transformed with *vgb* showed improved overall growth rate, faster germination and flowering, and increased productivity of certain oxygen-requiring metabolic pathways. VHb overexpressed in cabbage increased its submergence tolerance (Li et al. 2005). VHb-expressing hairy root lines of *Hyoscyamus muticus* displayed improved growth properties and accumulated hyoscyamine production (Wilhelmson et al. 2005). Transgenic tobacco plants

with *vgb* exhibited enhanced growth, and contained 30-40% more chlorophyll and 34% more nicotine than controls (Holmberg et al. 1997). Suspension cell cultures generated from VHb-expressing tobacco plants did not show any lag-phase and exhibited improved cell growth (Farres et al. 2002). These results boosted the present investigation of transformation of *vgb* into *E. ulmoides* hairy roots in order to enhance accumulation of secondary metabolites.

4.2 Objective

To transform VHb into *E. ulmoides* hairy roots and investigate its effect on the growth and secondary metabolites of the hairy roots.

4.3. Materials and Methods

4.3.1. Plasmids

Plasmid pGEM-VHb and plamid pBI 121 were bestowed by Dr. Zhibi Hu at Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, P.R. China.

4.3.2. Reconstruction of Binary Vector

After PCR amplification and double digestion by restriction endonucleases, *Xba*I and *Sac*I, the open reading frame (ORF) of *vgb* was inserted into plasmid pBI 121 and formed the reconstructed plasmid pBI-VHB. Consequently, the constructed plasmid pBI-VHB was introduced into competent cells of *E. coli* DH5α. Transformants were screened by kanamycin resistance and subsequently characterized by PCR and restriction endonuclease analysis. The plasmid pBI-VHB, kept in *E. coli* DH5α, was then mobilized into *A. rhizogenes* LBA9402 with the helper plasmid, pRK2013 stored in *E. coli* DH5α. A positive clone, after confirmation of the presence of *vgb* gene by PCR and enzymatic digestion analysis, was used to transform the leaves of *E. ulmoides*.

4.3.3. Molecular Identification

4.3.3.1. PCR Characterization

Genomic DNA was extracted from fresh hairy roots and purified according to acetyl trimethyl ammonium bromide (CTAB) method (Sambrook etal, 1989). A pair of primers, 5'-GATATATGCCAAATTTACACTAG-3' and 5'-GTTAACAAAGTAGGAAACAGG-3', was applied to amplify a 557 bp fragment of *rolC* gene. Another pair of primers, 5'-TTAGACCAGCAAACCATTAACATC-3' and 5'-TTATTCAACCGCTTGAGCGTAC-3' was employed to amplify a 438 bp fragment of *vgb* gene. The 20 µl PCR reaction mixture contained 1 unit of *Taq* DNA polymerase (Promega, Madison, WI), 0.5 µl of 10 mM dNTPs, 2 µl of 10×PCR buffer, 1.6 µl of 25 mM $MgCl_2$, 2 µl of each primer (5 µM) and 100 ng of genomic DNA. PCR was conducted under the following conditions: 94 ºC pre-denaturation for 5 min; 94 ºC denaturation for 0.5 min, 56 ºC annealing for 0.5 min, 72 ºC extending 1 min for 30 cycles; followed by an extra extending cycle of 7 min. Finally, 5 µl of each amplified product was detected by electrophoresis on 1.2% agarose gel.

4.3.3.2. Southern and Northern Blotting

After purification by phenol and quantification by measuring OD value at 260 nm, total of 15 µg genomic DNA of each hairy root line was digested with *Xba*I overnight and then transferred onto a Hybond^{TM} N^+ nylon membrane (Amersham, Piscataway, NJ), followed by UV cross-linking to fix the DNA on the membrane. The ORF of *vgb* gene, ca. 400 bp, was used as template for randomized primer labeling and hybridized with the blot according to the manual of DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Indianapolis, IN). Signals were visualized by chemical and immunological detection methods within the same kit.

Total RNA was isolated by using Rneasy Plant Mini Kit (Qiagen, Valencia, CA). The quality and quantity of the RNA was detected by running a 1.2% formaldehyde denatured

agarose gel and measuring the optimal density values at 260 nm and 280 nm, respectively. 25 μg of total RNA of each hairy root line was loaded on 1.0% formaldehyde denatured agarose gel and transferred onto a Hybond^{TM} N^+ nylon membrane (Amersham, Piscataway, NJ) after electrophoresis. Consequently, RNA was fixed on the membrane by UV cross-linking. The same probe of *vgb* labeled for Southern blotting was used to detect the expression of *vgb* in different transgenic hairy root lines.

4.3.4. Hairy Root Culture

Hairy root transformed with VHb was cultured under modified culture condition, i.e. in MS basal medium supplied with 1 μM IBA and 4.5% sucrose, and compared with normal hairy roots cultured under the same condition. About 0.5 g of the fresh hairy roots were inoculated into 125 ml Erlenmeyer flasks containing 50 ml new liquid media and cultured continuously on an orbital shaker at 100 rpm and 25ºC in the dark for four weeks.

4.3.5. Chemical Extraction and HPLC Analysis

After harvesting, hairy roots were freeze-dried immediately and ground into powder. For GA and PG analyses, approximately 0.1 g of each sample was extracted with 1.5 ml 70% (v/v) methanol for three days. Then the extracts were subjected to centrifugation at 12,000 rpm. The supernatant was collected and blown to dry. The extraction procedure was conducted twice and the dried extracts were combined, dissolved in 1 ml distilled water and purified by C18 SPE column. The eluent of methanol was collected, dried, followed by dissolving in 0.3 ml 30% (v/v) methanol and filtered through a 0.2 μm syringe filter prior to HPLC analysis. For CA analysis, approximately 50 mg of each sample was extracted twice with 1 ml deionized water in a boiling water-bath for 1 hr. The extracts were then subjected to centrifugation at 12, 000 rpm. The supernatant was collected and freeze-dried. After that, the freeze-dried extracts were dissolved in 250 μl sterile water and filtered through 0.2 μm syringe filter before HPLC analysis.

HPLC analysis was conducted on a Waters 600 system with a 717 autosampler and a 2996 photodiode array detector at ambient temperature. The system was computer controlled and analyzed with the Empower software system. Separation was carried out using a Symmetry C18 column (5.0 μm, 150×4.6 mm I.D.) with a guard cartridge (5.0 μm, 20×3.9 mm I.D.). For GA and PG analyzes, the mobile phase was set as below: 0～10 min, MeOH-0.15% acetic acid/10:90; 10～40 min, gradient from MeOH-0.15% acetic acid/ 10:90 to 40:60; 40～50 min, MeOH. The flow rate was 1.0 ml/min and detection wavelength was set at 236 nm for GA and 226 nm for PG. The retention time of GA was 12.1 minute and that of PG was 37.8 minute. The analysis of CA was performed with a mixture of 0.15% (v/v) acetic acid and acetonitrile (92:8) as mobile phase at a flow rate of 1 ml min^{-1}. The detection wavelength of CA was 325 nm with retention time at 11.2 min. The calibration curve for GA is as follows: Y=1229325*X-36276 with R^2=0.99990 and linear range: 0.315~ 1.575 μg. The calibration curve for PG is as follows: Y= 1536588*X -20592 with R^2=0.99995 and linear range: 0.180~0.900 μg. For CA, the calibration curve is Y=4263632*X -411363 with R^2=0.99826 and linear range: 0.165~2.0625μg. For all the calibration curves, X represents amount (μg) and Y stands for peak area.

4.3.6. Statistical Analysis

All the data were collected and subjected to ANOVA analysis by using the MIXED program of SAS software. Tukey adjustment was used for all pairwise comparisons.

4.4. Results

4.4.1. Construction of Plamid pBI-VHb

Plasmid pBI-VHb was constructed by replacing GUS gene between *Xba*I and *Sac*I sites in plasmid pBI 121. Consequently, the new constructed plasmid was supplied a 35s CaMV promoter and a Nos terminator to *vgb*, which guarantee the strong expression of *vgb* within plant cells (Fig 4.4.1). After that, plasmid pBI-VHb was mobilized into *A. rhizogenes*

LBA 9402 by triparental mating approach. The positive *A. rhizogenes* strain, namely LBA 9402-VHb was used for transformation.

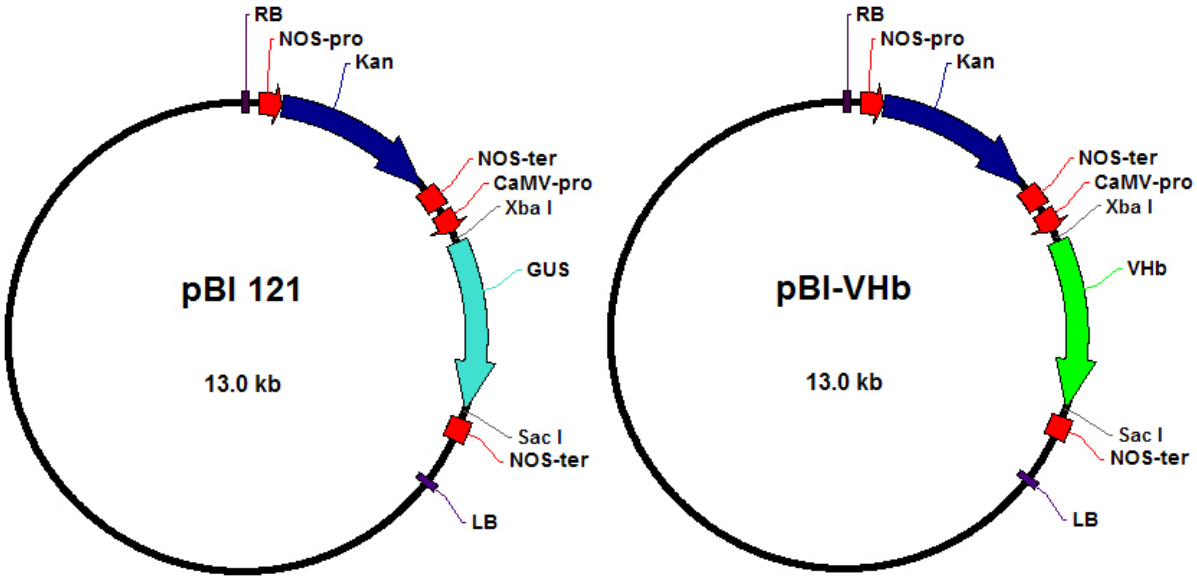


Fig 4.4.1. Structures of plasmid pBI 121 and pBI-VHb.

4.4.2. Molecular Identification

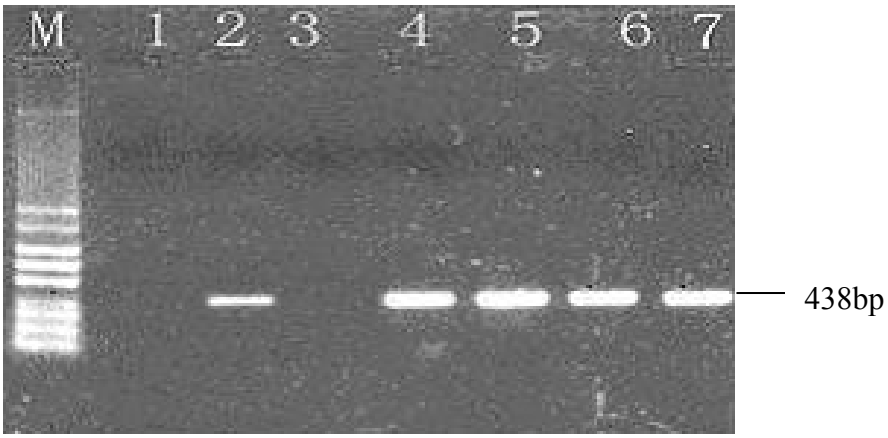


Fig 4.4.2.1. PCR analysis of genomic DNA from VHb hairy root lines and normal hairy root of *E. ulmoides* to amplify a 438 bp fragment of *vgb* gene. Lanes: M, 100 bp DNA marker; 1, Sterile water used as negative control for PCR; 2, Plasmid pGEM-VHb used as positive control for PCR; 3, Normal hairy root; 4-7: Hairy roots transformed with VHb.

Four hairy root lines induced by LBA 9402-VHb survived kanamycin screening and were subjected to further molecular identification. PCR result showed that *vgb* was transferred into all the four hairy root lines (Fig 4.4.2.1). Southern blotting further corroborated the integration of VHb gene into the genomic DNA of hairy root lines (Fig

4.4.2.2). However, Northern blotting revealed that *vgb* only expressed in one hairy root line (Fig 4.4.2.3). Thus the positive hairy root line, i.e. hairy root line expressing VHb gene, was used for further study.

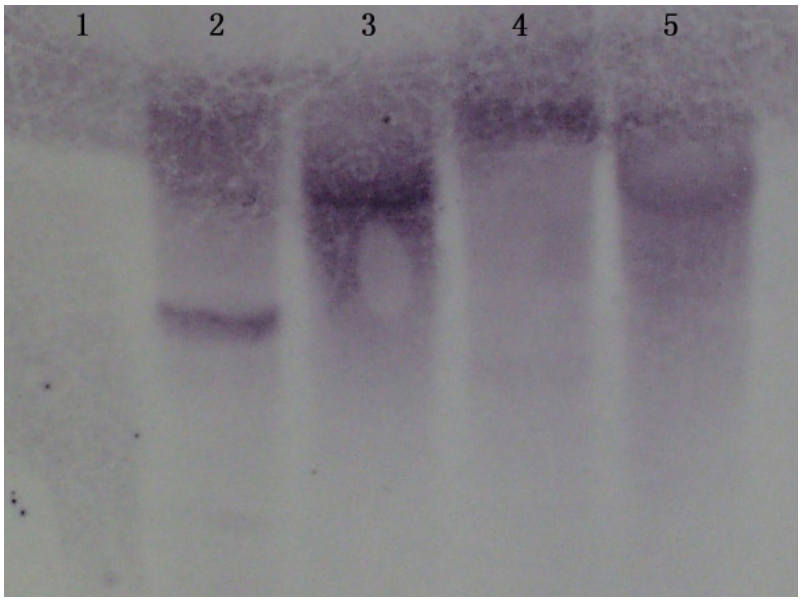


Fig 4.4.2.2. Southern-blot hybridization analysis of genomic DNA isolated fromVHb hairy root lines and normal hairy root of *E. ulmoides*. DNA samples (15 μg) were digested with *Xba* I, and hybridized with digoxigenin-labeled *vgb* gene probe. Lanes: 1, DNA from normal hairy root; 2-5, Hairy roots transformed with VHb.

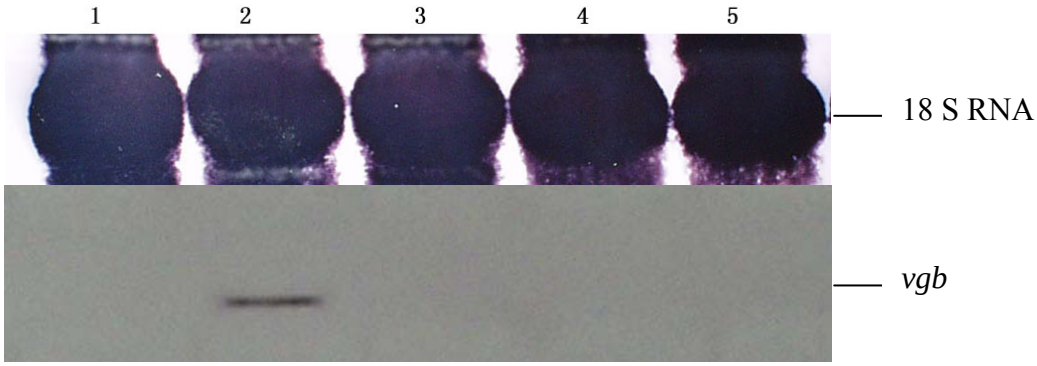


Fig 4.4.2.3. Northern-blot hybridization analysis of RNA isolated from VHb hairy root lines and normal hairy root of *E. ulmoides*. RNA samples (25 μg) were hybridized with digoxigenin-labeled *vgb* gene and 18 S RNA probes, respectively. Lanes: 1, Normal hairy root; 2-5, Hairy roots transformed with VHb.

4.4.3. Growth and Chemical Analysis

4.4.3.1. Growth Comparison

Results showed that both fresh and dry mass of VHb hairy roots were significantly higher than that of normal hairy roots (Fig 4.4.3.1). The dry mass of VHb hairy roots

increased 19.5% than that of normal hairy roots, which indicated the expression of VHb accelerated the growth of hairy roots.

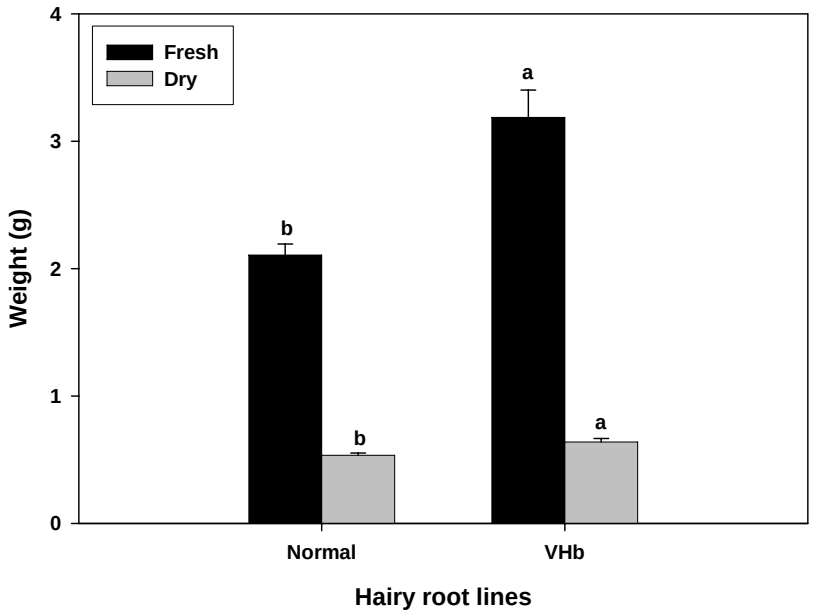


Fig 4.4.3.1. Growth comparison between normal and VHb hairy roots of *E. ulmoides*. Vertical bars represent standard error. Each treatment is replicated nine times. Significant differences between treatments are labeled by different letters (α = 0.05).

4.4.3.2. Chemical Analysis

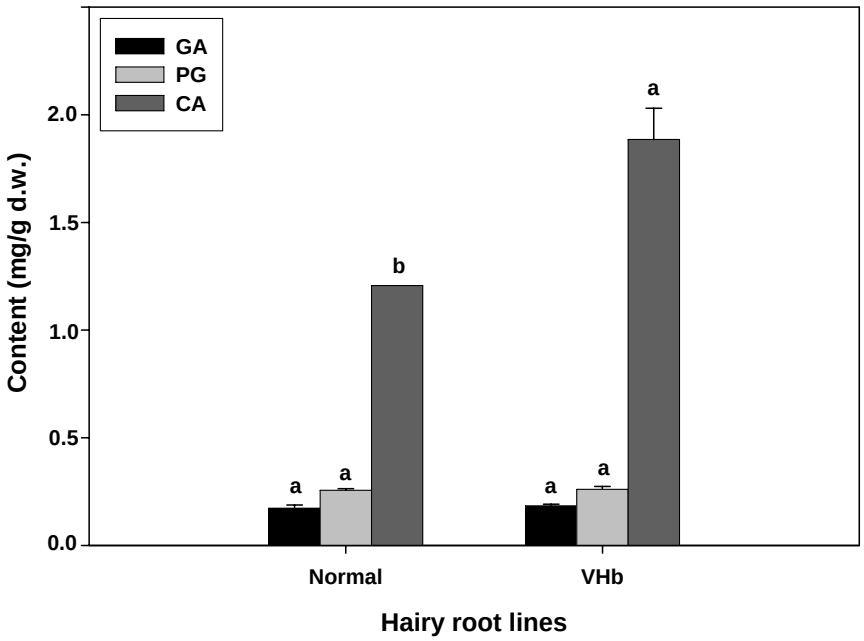


Fig 4.4.3.2.1. Comparison of contents of GA, PG and CA between normal and VHb hairy roots of *E. ulmoides*. Vertical bars represent standard error. Each treatment is replicated nine times. Significant differences between treatments are labeled by different letters (α = 0.05).

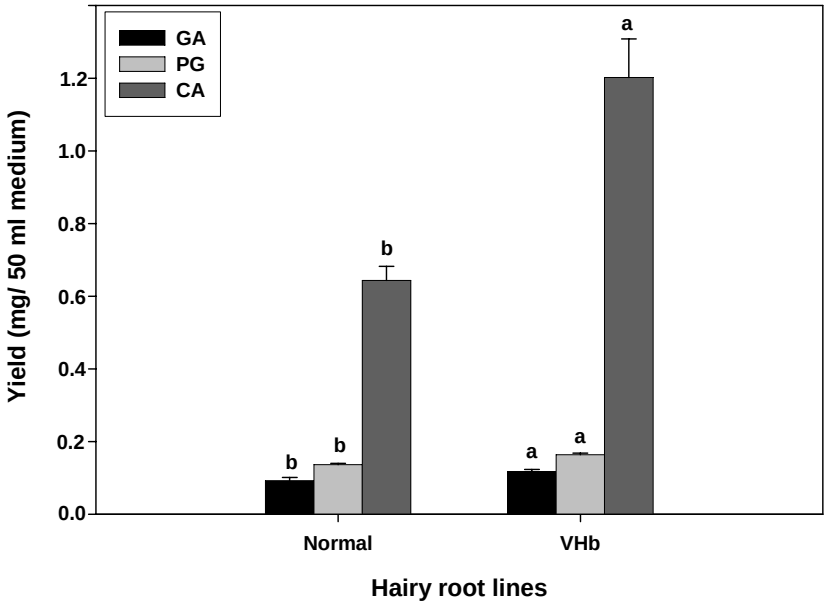


Fig 4.4.3.2.2. Comparison of yields of GA, PG and CA between normal and VHb hairy roots of *E .ulmoides*. Vertical bars represent standard error. Each treatment is replicated nine times. Significant differences between treatments are labeled by different letters ($\alpha = 0.05$).

Chemical analyses displayed that the contents of three compounds, i.e. GA, PG and CA, in VHb hairy roots were higher than that in normal hairy roots (Fig 4.4.3.2.1). However, only the difference in CA content was significant, while the difference in GA and PG contents was not so remarkable. As the yields of the three compounds were compared, the differences between VHb hairy roots and normal hairy roots were significant (Fig 4.4.3.2.2). The yield of GA in VHb hairy roots increased 26.7% and achieved 0.117 mg/flask, while the yield of PG increased 20.0% and attained 0.164 mg/flask. The augmentation of the yield of CA was extremely high, which increased 86.8% and reached 1.202 mg/flask.

4.5. Discussions

Hairy root cultures are difficult to scale up, which limits their commercial viability. A characteristic of hairy root growth is the formation of root clumps, which develop into a tight matrix, remaining essentially stationary in a bioreactor and causing poor oxygen and nutrient transfer (Yu et al. 1994). Oxygen deficiency has been shown to be a limiting factor for the growth and biomass accumulation of hairy roots (Kanokwaree et al. 1997). Studies have

displayed that VHb-expression could improve the growth of cells, enhance plant resistance to hypoxia and flooding, speed germination and flowering (Bulow et al. 1999; Farres et al. 2002; Mao et al. 2003). Recent studies demonstrated VHb transformation improved the growth of *H. muticus* hairy roots in shake flask cultures (Wilhelmson et al. 2005). Studis also revealed that VHb expression could enhance nicotine accumulation in tobacco and hyoscyamine production in hairy roots (Holmberg et al. 1997; Wilhelmson et al. 2005). However, the effects of VHb on the growth and secondary metabolites of *E. ulmoides* hairy roots are not known.

Our experiments revealed that VHb expression significantly improved the growth of *E. ulmoides* hairy roots. Furthermore, the content of CA in transformed hairy roots was enhanced remarkably and was similar to the response of hairy roots to increasing of sucrose concentration in the medium. It was well known that sucrose and oxygen were involved in glycolysis. Thus the increasing of sucrose concentration or oxygen supplementation should have the same effect on CA production. The contents of GA and PG were also increased but not in a statistically significant manner. Finally, the yields of the three compounds were improved markedly compared with the control hairy roots. The results indicated that the pathway of CA synthesis was possibly associated with effective intracellular oxygen concentration and thus carbohydrate metabolism, while the metabolism of GA and PG synthesis was not relevant.

4.6. Summary

VHb gene was inserted between *Xba* I and *Sac* I sites of plasmid pBI 121 and formed a new plasmid pBI-VHb. The new constructed plasmid was introduced into *A. rhizogenes* LBA 9402 and employed for transformation. After molecular identification, i.e. PCR, Southern blotting and Northern blotting, the positive hairy root line was subcultured for further investigation. Growth studies showed that VHb hairy roots accumulated more

biomass than normal hairy roots. Chemical analysis demonstrated that VHb hairy roots produced more GA, PG and CA than normal hairy roots. These results indicated that VHb-expression could improve growth and enhance synthesis of secondary metabolites of *E. ulmoides* hairy roots.

CHAPTER 5. CONCLUSIONS AND FUTURE RESEARCH

From the whole study, we draw following conclusions:

I.Hairy root culture system of *E. ulmoides* had been established successfully.

II. Culture conditions had varied effects on the growth and secondary metabolites of *E. ulmoides* hairy roots.

III. Heterologous expression of VHb in *E. ulmoides* hairy roots could improve growth and enhance synthesis of secondary metabolites of *E. ulmoides* hairy roots

Our future research will focus on following aspects:

I. Isolation new compounds from *E. ulmoides* hairy roots based on chemical fingerprints.

II. Scale-up culture technique study of *E. ulmoides* hairy roots.

III. Pharmacological study of *E. ulmoides* hairy roots.

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APPENDIX A. SAS PROGRAMS FOR ANOVA ANALYSIS

SAS program for analysis of auxin type on growth of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
data auxin1;
 set work.auxin(keep=clone auxin AdjHarWeight AdjDryWeight);
 run;
proc mixed data=auxin1;
class clone auxin;
model AdjHarWeight=clone auxin;
run;
proc mixed data=auxin1;
class auxin;
model AdjHarWeight=auxin;
lsmeans auxin/adj=tukey;
run;
proc mixed data=auxin1;
class clone auxin;
model AdjHarWeight=auxin;
random clone;
title 'AdjHarWeight ANOVA analysis';
run;
proc mixed data=auxin1;
class clone auxin;
model AdjDryWeight=clone auxin;
run;
proc mixed data=auxin1;
class  auxin;
model AdjDryWeight=auxin;
lsmeans auxin/adj=tukey;
run;
proc mixed data=auxin1;
class clone auxin;
model AdjDryWeight=auxin;
random clone;
lsmeans auxin/adj=tukey;
title 'Dry Weight ANOVA analysis';
run;
proc sort data=auxin1 out=auxin2;
by auxin;
run;
proc means data=auxin2 mean stderr noprint;
by auxin;
var AdjHarWeight AdjDryWeight;
output out=auxin3 mean=m1-m7 stderr=s1-s7;
run;
proc print;
run;
proc mixed data=work.auxin;
class clone auxin;
model DFRatio=auxin;
random clone;
lsmeans auxin/adj=tukey;
run;
proc sort data=work.auxin out=auxin4;
by auxin;
run;
proc means data=auxin4 mean stderr noprint ;
by auxin;
```

```
var DFRatio;
output out=auxin5 mean=m1-m7 stderr=s1-s7;
run;
proc print;
run;
```

SAS program for analysis of growth curve of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter nonumber;
data growth1;
 set growth;
run;
proc sort data=growth1 out=growth3;
by clone;
run;
proc mixed data=growth3;
class clone;
model AdjHarWeight=clone;
run;

proc sort data=growth1 out=growth2;
by HarTime;
run;
proc means mean stderr data=growth2 ;
by HarTime;
var AdjHarWeight AdjDryWeight;
run;
proc univariate data=growth2;
var AdjHarWeight AdjDryWeight;
run;

proc mixed data=growth;
class clone HarTime;
model DFRatio=HarTime;
random clone;
lsmeans HarTime/adj=tukey;
run;
proc sort data=growth;
 by HarTime;
 run;
proc means mean stderr data=growth;
 by HarTime;
 var DFRatio;
run;
```

SAS program for analysis of medium type and light on the growth of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
proc mixed data=Med_lig;
class clone Medium lightcondition;
model AdjHarWeight=Medium lightcondition Medium*lightcondition clone;
run;
proc mixed data=Med_lig;
class clone Medium lightcondition;
model AdjHarWeight=Medium lightcondition Medium*lightcondition;
random clone;
lsmeans Medium lightcondition/adj=tukey;
run;
proc mixed data=Med_lig;
```

```
class clone Medium lightcondition;
model AdjDryWeight=Medium lightcondition Medium*lightcondition clone;
run;
proc mixed data=Med_lig;
class clone Medium lightcondition;
model AdjDryWeight=Medium lightcondition Medium*lightcondition;
random clone;
run;
proc mixed data=Med_lig;
class Medium lightcondition;
model AdjDryWeight=medium lightcondition Medium*lightcondition;
lsmeans Medium lightcondition/adj=tukey;
run;
proc sort data=Med_lig out=Med_lig1;
by Medium;
run;
proc means data=Med_lig1 mean stderr noprint ;
 by Medium;
 var AdjHarWeight AdjDryWeight;
 output out=med_lig2 mean=m1-m3 stderr=s1-s3;
run;
proc print;
run;
proc sort data=Med_lig out=Med_lig3;
by lightcondition;
run;
proc means  data=Med_lig3 mean stderr noprint;
 by lightcondition;
 var AdjHarWeight AdjDryWeight;
 output out=med_lig4 mean=m1-m2 stderr=s1-s2;
run;
proc print;
run;
```

SAS program for analysis of medium strength on the growth of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
data medium_strength1;
 set strength (keep=clone strength AdjHarWeight AdjDryWeight);
run;
proc mixed data=medium_strength1;
class clone strength;
model AdjHarWeight=clone strength;
run;
proc mixed data=medium_strength1;
class  strength;
model AdjHarWeight= strength;
lsmeans strength/adj=tukey;
run;
proc mixed data=medium_strength1;
class clone strength;
model AdjDryWeight=clone strength;
run;
proc mixed data=medium_strength1;
class strength;
model AdjDryWeight= strength;
lsmeans strength/adj=tukey;
run;

proc sort data=medium_strength1 out=medium_strength2;
```

```
by strength;
run;
proc means data=medium_strength2 mean stderr noprint;
by strength;
var AdjHarWeight AdjDryWeight;
output out=medium_strength3 mean=m1-m4 stderr=s1-s4;
run;
proc print;
run;
```

SAS program for analysis the growth and secondary metabolites of *E. ulmoides* hairy roots cultured under modified condition.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.NEWGROWTH
            DATAFILE= "F:\hairy root culture\New growth curve.xls"
            DBMS=EXCEL2000 REPLACE;
     GETNAMES=YES;
RUN;
proc mixed data=newgrowth;
class clone week;
model AdjDryWeight=week;
random clone;
lsmeans week/adj=tukey;
run;
proc sort data=newgrowth out=newgrowth1;
by week;
run;
proc means data=newgrowth1;
by week;
var AdjDryWeight;
output out=newgrowth2 mean=m1-m6 stderr=s1-s6;
run;
proc print;
run;
/*Fresh Weight Comparison*/
proc mixed data=newgrowth;
class clone week;
model AdjHarWeight=week;
random clone;
lsmeans week/adj=tukey;
run;
proc sort data=newgrowth out=newgrowth3;
by week;
run;
proc means data=newgrowth3;
by week;
var AdjHarWeight;
output out=newgrowth3 mean=m1-m6 stderr=s1-s6;
run;
proc print;
run;
/*GA and PG analysis*/
proc sort data=newgrowth out=newgrowth4;
by week;
run;
proc means data=newgrowth4 noprint;
by week;
var GAConcen PGConcen GAProductivity PGProductivity;
output out=newgrowth4 mean=m1-m6 stderr=s1-s6;
```

```
run;proc print; run;
proc mixed data=newgrowth;
class clone week;
model GAConcen=week;
random clone;
lsmeans week/adj=tukey;
run;
/*for ca analysis*/
proc mixed data=newgrowth;
class clone week;
model CAConcen=week;
random clone;
lsmeans week/adj=tukey;
run;
proc mixed data=newgrowth;
class clone week;
model CAYield=week;
random clone;
lsmeans week/adj=tukey;
run;
proc sort data=newgrowth out=newgrowth4;
by week;
run;
proc means data=newgrowth4 noprint;
by week;
var CAConcen CAYield;
output out=newgrowth4 mean=m1-m6 stderr=s1-s6;
run;proc print; run;
```

SAS program for analysis of the initial pH on the growth of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
data pH2;
  set Ph (keep=clone pH AdjHarWeight AdjDryWeight);
run;
proc mixed ;
class clone pH;
model AdjHarWeight=clone pH/solution;
lsmeans pH/adj=tukey;
run;
proc mixed ;
class clone pH;
model AdjHarWeight=pH /solution;
random clone;
lsmeans pH/adj=tukey;
run;
proc mixed ;
class clone pH;
model AdjDryWeight=clone pH/solution;
lsmeans pH/adj=tukey;
run;
proc mixed;
class clone pH;
model AdjDryWeight=pH/solution;
random clone;
lsmeans pH/adj=tukey;
run;
proc sort data=pH2 out=pH3;
by pH;
run;
```

```
proc means data=pH3 mean stderr noprint;
by pH;
var AdjHarWeight AdjDryWeight;
output out=pH4 mean=m1-m6 stderr=s1-s6;
run;
proc print;
run;
```

SAS program for analysis of growth comparison among different hairy root lines of *E. ulmoides*.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
proc mixed data=rootlines;
class clone;
model AdjDryWeight=clone;
lsmeans clone/adj=tukey;
run;
proc sort data=rootlines out=rootlines1;
by clone;
run;
proc means data=rootlines1 mean stderr noprint;
by clone;
var AdjDryWeight;
output out=rootlines2 mean=m1-m8 stderr=s1-s8;
run;
proc print;
run;
/*combine all the non-transgenetic lines*/
proc mixed data=rootlines;
class clone2;
model AdjDryWeight=clone2;
lsmeans clone2/adj=tukey;
run;
proc sort data=rootlines out=rootlines3;
by clone2;
run;
proc means data=rootlines3 mean stderr noprint;
by clone2;
var AdjDryWeight;
output out=rootlines4 mean=m1-m2 stderr=s1-s2;
run;
proc print;
run;
/*Fresh Weight Hairy roots*/
proc mixed data=rootlines;
class clone;
model AdjHarWeight=clone;
lsmeans clone/adj=tukey;
run;
proc sort data=rootlines out=rootlines1;
by clone;
run;
proc means data=rootlines1 mean stderr noprint;
by clone;
var AdjHarWeight;
output out=rootlines2 mean=m1-m8 stderr=s1-s8;
run;
proc print;
run;
/*combine all the non-transgenetic lines*/
```

```
proc mixed data=rootlines;
class clone2;
model AdjHarWeight=clone2;
lsmeans clone2/adj=tukey;
run;
proc sort data=rootlines out=rootlines3;
by clone2;
run;
proc means data=rootlines3 mean stderr noprint;
by clone2;
var AdjHarWeight;
output out=rootlines4 mean=m1-m2 stderr=s1-s2;
run;
proc print;
run;
/*without clone vhb4*/
PROC IMPORT OUT= WORK.NEW
            DATAFILE= "F:\hairy root culture\Comparison among  transgeni
c hairy roots-2.xls"
            DBMS=EXCEL REPLACE;
     SHEET="Sheet1$";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
RUN;
proc mixed data=new;
class clone;
model AdjDryWeight=clone;
lsmeans clone/adj=tukey;
run;
proc sort data=new out=new1;
by clone;
run;
proc means data=new1 mean stderr noprint;
by clone;
var AdjDryWeight;
output out=new2 mean=m1-m7 stderr=s1-s7;
run;
proc print;
run;
/*combine all the non-transgenetic lines*/
proc mixed data=new;
class clone2;
model AdjDryWeight=clone2;
lsmeans clone2/adj=tukey;
run;
proc sort data=new out=new3;
by clone2;
run;
proc means data=new3 mean stderr noprint;
by clone2;
var AdjDryWeight;
output out=new4 mean=m1-m2 stderr=s1-s2;
run;
proc print;
run;
```

SAS program for analysis of sucrose concentration and medium strength on the growth of *E. ulmoides* hairy roots.

```
Dm 'log;clear;output;clear';
options nodate nocenter nonumber;
data salt1;
 set Salt_sucrose;
run;
proc mixed;
 class clone Nutrient Sucrose;
 model AdjHarWeight=clone Nutrient Sucrose Nutrient*Sucrose;
 lsmeans Nutrient Sucrose Nutrient*Sucrose/ adj=tukey;
run;
proc mixed;
 class clone Nutrient Sucrose;
 model AdjDryWeight=clone Nutrient Sucrose Nutrient*Sucrose;
 lsmeans Nutrient Sucrose Nutrient*Sucrose/ adj=tukey;
run;
proc sort out=salt2;
 by Nutrient;
run;
proc means data=salt2 mean stderr noprint;
 by Nutrient;
 var AdjHarWeight AdjDryWeight;
 output out=salt3 mean=m1-m3 stderr=s1-s3;
run;
proc print data=salt3;
run;
proc sort data=salt1 out=salt4;
 by Sucrose;
run;
proc means data=salt4 mean stderr noprint;
 by Sucrose;
 var AdjHarWeight AdjDryWeight;
 output out=salt5 mean=m1-m3 stderr=s1-s3;
run;
proc print data=salt5;
run;
proc sort data=salt1 out=salt6;
by Nutrient Sucrose;
run;
proc means mean stderr noprint;
 by Nutrient Sucrose;
 var AdjHarWeight AdjDryWeight;
 output out=salt7 mean=m1-m9 stderr=s1-s9;
 run;
 proc print;
 run;
```

SAS program for analysis of culture volume on the growth of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
proc mixed data=work.volume;
class Clone volume;
model IncreaseRatio= Clone volume;
lsmeans volume/adj=tukey;
run;
proc mixed data=work.volume;
class Clone volume;
model IncreaseRatio=volume;
random Clone;
```

```
lsmeans volume/adj=tukey;
run;
proc sort data=work.volume out=volume1;
by volume;
run;
proc means data=volume1 mean stderr noprint ;
by volume;
var IncreaseRatio;
output out=volume2 mean=m1-m4 stderr=s1-s4;
run;
proc print;
run;
```

SAS program for analysis of auxin on chemical components of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
proc mixed data=auxin;
class samplename subcode;
model GAConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;

proc mixed data=auxin;
class samplename subcode;
model PGConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=auxin;
class samplename subcode;
model CAConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=auxin;
class samplename subcode;
model GAYield=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=auxin;
class samplename subcode;
model PGYield=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=auxin;
class samplename subcode;
model CAYield=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc sort data=auxin out=auxin1;
by samplename;
run;

proc means data=auxin1 noprint ;
var GAConcen PGConcen CAConcen GAYield PGYield CAYield;
by samplename;
```

```
output out=auxin2 mean=m1-m7 stderr=s1-s7;
run;
proc print; run;
```

SAS program for analysis of chemical components of different hairy root lines of *E. ulmoides*.

```
Dm 'output;clear;log;clear';
options nodate nocenter nonumber;
proc mixed data=differline;
class samplename;
model GAConcen=samplename;
lsmeans samplename/adj=tukey;
run;
proc mixed data=differline;
class samplename;
model PGConcen=samplename;
lsmeans samplename/adj=tukey;
run;
proc mixed data=differline;
class samplename;
model CAConcen=samplename;
lsmeans samplename/adj=tukey;
run;
proc mixed data=differline;
class samplename;
model GAYield=samplename;
lsmeans samplename/adj=tukey;
run;
proc mixed data=differline;
class samplename;
model PGYield=samplename;
lsmeans samplename/adj=tukey;
run;
proc mixed data=differline;
class samplename;
model CAYield=samplename;
lsmeans samplename/adj=tukey;
run;
proc sort data=differline out=differline1;
by samplename;
run;
proc means data=differline1 noprint;
var GAConcen PGConcen CAConcen GAYield PGYield CAYield;
by samplename;
output mean=m1-m8 stderr=s1-s8;
run;
proc print; run;
```

SAS program for analysis of light elicitation on normal hairy roots of *E. ulmoides*.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
proc mixed data=lightelicit1;
class samplename subcode;
model GAConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=lightelicit1;
class samplename subcode;
model GAYield=samplename;
```

```
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=lightelicit1;
class samplename subcode;
model PGConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=lightelicit1;
class samplename subcode;
model PGYield=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=lightelicit1;
class samplename subcode;
model CAConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=lightelicit1;
class samplename subcode;
model CAYield=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc sort data=lightelicit1 out=lightelicit2;
by samplename;
run;
proc means data=lightelicit2 noprint;
var GAConcen GAYield PGConcen PGYield CAConcen CAYield;
by samplename;
output out=lightelicit3 mean=m1-m6 stderr=s1-s6;
run;
proc print;run;
```

SAS program for analysis of light elicitation on VHb hairy roots of *E. ulmoides*.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
proc mixed data=lightelicit;
class samplename source;
model GAConcen=samplename source;
lsmeans samplename/adj=tukey;
run;
proc mixed data=lightelicit;
class samplename source;
model GAYield=samplename source;
lsmeans samplename/adj=tukey;
run;
proc mixed data=lightelicit;
class samplename source;
model PGConcen=samplename source;
lsmeans samplename/adj=tukey;
run;
proc mixed data=lightelicit;
class samplename source;
model PGYield=samplename source;
lsmeans samplename/adj=tukey;
run;
```

```
proc mixed data=lightelicit;
class samplename source;
model CAConcen=samplename source;
lsmeans samplename/adj=tukey;
run;
proc mixed data=lightelicit;
class samplename source;
model CAYield=samplename source;
lsmeans samplename/adj=tukey;
run;
proc sort data=lightelicit out=lightelicit2;
by samplename;
run;
proc means data=lightelicit2 noprint;
var GAConcen GAYield PGConcen PGYield CAConcen CAYield;
by samplename;
output out=lightelicit3 mean=m1-m6 stderr=s1-s6;
run;
proc print;run;
proc sort data=lightelicit out=lightelicit2;
by source;
run;
proc means data=lightelicit2 noprint;
var GAConcen GAYield PGConcen PGYield CAConcen CAYield;
by source;
output out=lightelicit3 mean=m1-m6 stderr=s1-s6;
run;
proc print;run;
```

SAS program for analysis of medium type and light on chemical components of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
proc mixed data=medium_light;
class medium light subcode;
model GAConcen=medium light medium*light;
random subcode;
lsmeans medium light medium*light/adj=tukey;
run;
proc mixed data=medium_light;
class medium light subcode;
model PGConcen=medium light medium*light;
random subcode;
lsmeans medium light medium*light/adj=tukey;
run;
proc mixed data=medium_light;
class medium light subcode;
model GAYield=medium light medium*light;
random subcode;
lsmeans medium light medium*light/adj=tukey;
run;
proc mixed data=medium_light;
class medium light subcode;
model PGYield=medium light medium*light;
random subcode;
lsmeans medium light medium*light/adj=tukey;
run;
proc sort data=medium_light out=medium_light2;
by medium;
run;
```

```
proc means data=medium_light2 noprint;
var GAConcen PGConcen GAYield PGYield;
by medium;
output out=medium_light3 mean=m1-m4 stderr=s1-s4;run;
proc print; run;
proc sort data=medium_light out=medium_light3;
by light;
run;
proc means data=medium_light3 noprint;
var GAConcen PGConcen GAYield PGYield;
by light;
output out=medium_light4 mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
proc mixed data=medium_light;
class medium light subcode;
model CAConcen=medium light medium*light;
random subcode;
lsmeans medium light medium*light/adj=tukey;
run;
proc mixed data=medium_light;
class medium light subcode;
model CAYield=medium light medium*light;
random subcode;
lsmeans medium light medium*light/adj=tukey;
run;
proc sort data=medium_light out=medium_light2;
by medium;
run;
proc means data=medium_light2 noprint;
var CAConcen CAYield ;
by medium;
output out=medium_light3 mean=m1-m4 stderr=s1-s4;run;
proc print; run;
proc sort data=medium_light out=medium_light3;
by light;
run;
proc means data=medium_light3 noprint;
var CAConcen CAYield;
by light;
output out=medium_light4 mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
```

SAS program for analysis of medium strength on chemical components of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
proc mixed data=mediumstren;
class samplename subcode;
model GAConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=mediumstren;
class samplename subcode;
model PGConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
```

```
proc mixed data=mediumstren;
class samplename subcode;
model GAYield=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=mediumstren;
class samplename subcode;
model PGYield=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc sort data=mediumstren out=mediumstren1;
by samplename;
run;
proc means data=mediumstren1 noprint;
var GAConcen PGConcen GAYield PGYield;
by samplename;
output mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
proc mixed data=mediumstren;
class samplename subcode;
model CAConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=mediumstren;
class samplename subcode;
model CAYield=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc sort data=mediumstren out=mediumstren1;
by samplename;
run;
proc means data=mediumstren1 noprint;
var CAConcen CAYield;
by samplename;
output mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
```

SAS program for analysis of sucrose concentration and medium strength on the chemical components of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.nutrient_sugar
            DATAFILE= "F:\hairy root culture\HPLC analysis result\nutrie
nt_sugar.xls"
            DBMS=EXCEL REPLACE;
     SHEET="Sheet1$";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
     USEDATE=YES;
     SCANTIME=YES;
RUN;
proc mixed data=nutrient_sugar;
class nutrient sugar subcode;
```

```
model GAConcen=nutrient sugar nutrient*sugar;
random subcode;
lsmeans nutrient sugar nutrient*sugar/adj=tukey;
run;
proc mixed data=nutrient_sugar;
class nutrient sugar subcode;
model PGConcen=nutrient sugar nutrient*sugar;
random subcode;
lsmeans nutrient sugar nutrient*sugar/adj=tukey;
run;
proc mixed data=nutrient_sugar;
class nutrient sugar subcode;
model GAYield=nutrient sugar nutrient*sugar;
random subcode;
lsmeans nutrient sugar nutrient*sugar/adj=tukey;
run;
proc mixed data=nutrient_sugar;
class nutrient sugar subcode;
model PGYield=nutrient sugar nutrient*sugar;
random subcode;
lsmeans nutrient sugar nutrient*sugar/adj=tukey;
run;
proc sort data=nutrient_sugar out=nutrient_sugar1;
by nutrient;
run;
proc means data=nutrient_sugar1 noprint;
var GAConcen PGConcen GAYield PGYield;
by nutrient;
output out=nutrient_sugar2 mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
proc sort data=nutrient_sugar1 out=nutrient_sugar3;
by sugar;
run;
proc means data=nutrient_sugar3 noprint;
var GAConcen PGConcen GAYield PGYield;
by sugar;
output out=nutrient_sugar4 mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
proc sort data=nutrient_sugar out=nutrient_sugar5;
by nutrient sugar;
run;
proc means data=nutrient_sugar5 noprint;
var PGYield;
by nutrient sugar;
output out=nutrient_sugar6 mean=m1-m4 stderr=s1-s4;
run;
proc print; run;
```

SAS program for analysis initial pH on chemical components of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
proc mixed data=pH;
class samplename subcode;
model GAConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=pH;
```

```
class samplename subcode;
model PGConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=pH;
class samplename subcode;
model GAYield=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=pH;
class samplename subcode;
model PGYield=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc sort data=pH out=pH1;
by samplename;
run;
proc means data=pH1 noprint;
var GAConcen PGConcen GAYield PGYield;
by samplename;
output out=pH2 mean=m1-m6 stderr=s1-s6;
run;
proc print;run;
proc mixed data=pH;
class samplename subcode;
model CAConcen=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc mixed data=pH;
class samplename subcode;
model CAYield=samplename;
random subcode;
lsmeans samplename/adj=tukey;
run;
proc sort data=pH out=pH1;
by samplename;
run;
proc means data=pH1 noprint;
var CAConcen CAYield;
by samplename;
output out=pH2 mean=m1-m2 stderr=s1-s2;
run;
proc print; run;
```

SAS program for analysis of sucrose concentration, pH and auxin interaction on chemical components of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
PROC IMPORT OUT= WORK.phauxinsugar
            DATAFILE= "F:\hairy root culture\HPLC analysis result\pH_Aux
in_sugar.xls"
            DBMS=EXCEL REPLACE;
     SHEET="Sheet1$";
     GETNAMES=YES;
     MIXED=NO;
     SCANTEXT=YES;
```

```
     USEDATE=YES;
     SCANTIME=YES;
RUN;
proc mixed data=pHauxinsugar;
class pH auxin sugar subcode;
model GAConcen=pH|sugar|auxin;
random subcode;
lsmeans pH|sugar|auxin/adj=tukey;
run;
proc mixed data=pHauxinsugar;
class pH auxin sugar subcode;
model PGConcen=pH|sugar|auxin;
random subcode;
lsmeans pH|sugar|auxin/adj=tukey;
run;
proc mixed data=pHauxinsugar;
class pH auxin sugar subcode;
model GAYield=pH|sugar|auxin;
random subcode;
lsmeans pH|sugar|auxin/adj=tukey;
run;
proc mixed data=pHauxinsugar;
class pH auxin sugar subcode;
model PGYield=pH|sugar|auxin;
random subcode;
lsmeans pH|sugar|auxin/adj=tukey;
run;
proc sort data=pHauxinsugar out=pHauxinsugar1;
by pH;
run;
proc means data=pHauxinsugar1 noprint;
var GAConcen PGConcen GAYield PGYield;
by pH;
output out=pHauxinsugar2 mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
proc sort data=pHauxinsugar out=pHauxinsugar1;
by auxin;
run;
proc means data=pHauxinsugar1 noprint;
var GAConcen PGConcen GAYield PGYield;
by auxin;
output out=pHauxinsugar2 mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
proc sort data=pHauxinsugar out=pHauxinsugar1;
by sugar;
run;
proc means data=pHauxinsugar1 noprint;
var GAConcen PGConcen GAYield PGYield;
by sugar;
output out=pHauxinsugar2 mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
proc sort data=pHauxinsugar out=pHauxinsugar1;
by auxin sugar;
run;
proc means data=pHauxinsugar1 noprint;
var GAYield;
by auxin sugar;
output out=pHauxinsugar3 mean=m1-m4 stderr=s1-s4;
```

```
run;
proc print; run;
proc mixed data=pHauxinsugar;
class pH auxin sugar subcode;
model CAConcen=pH|sugar|auxin;
random subcode;
lsmeans pH|sugar|auxin/adj=tukey;
run;
proc mixed data=pHauxinsugar;
class pH auxin sugar subcode;
model CAYield=pH|sugar|auxin;
random subcode;
lsmeans pH|sugar|auxin/adj=tukey;
run;
proc sort data=pHauxinsugar out=pHauxinsugar1;
by pH;
run;
proc means data=pHauxinsugar1 noprint;
var CAConcen CAYield;
by pH;
output out=pHauxinsugar2 mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
proc sort data=pHauxinsugar out=pHauxinsugar1;
by auxin;
run;
proc means data=pHauxinsugar1 noprint;
var CAConcen CAYield ;
by auxin;
output out=pHauxinsugar2 mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
proc sort data=pHauxinsugar out=pHauxinsugar1;
by sugar;
run;
proc means data=pHauxinsugar1 noprint;
var CAConcen CAYield;
by sugar;
output out=pHauxinsugar2 mean=m1-m4 stderr=s1-s4;
run;
proc print;run;
```

SAS program for analysis of SA on chemical components of *E. ulmoides* hairy roots.

```
Dm 'output;clear;log;clear';
options nodate nocenter pageno=1;
proc mixed data=sa;
class SampleName subcode;
model GAConcen=SampleName;
random subcode;
lsmeans SampleName/adj=tukey;
run;
proc mixed data=sa;
class SampleName subcode;
model PGConcen=SampleName;
random subcode;
lsmeans SampleName/adj=tukey;
run;
proc sort data=sa out=sa1;
by SampleName;
run;
```

```
proc means data=sa1 noprint;
var GAConcen PGConcen;
by SampleName;
output mean=m1-m6 stderr=s1-s6;
run;
proc print;
run;
proc mixed data=sa;
class SampleName subcode;
model CAConcen=SampleName;
random subcode;
lsmeans SampleName/adj=tukey;
run;
proc sort data=sa out=sa1;
by SampleName;
run;
proc means data=sa1 noprint;
var CAConcen;
by SampleName;
output mean=m1-m6 stderr=s1-s6;
run;
proc print;
run;
```

SAS program for analysis of MeJA on chemical components of *E. ulmoides* hairy roots.

```
Dm "output; clear;log;clear";
options nodate nocenter pageno=1;
proc mixed data=meja;
class Clone MeJAConcen;
model GAConcen=Clone MeJAConcen;
random Clone;
lsmeans MeJAConcen/adj=tukey;
run;
proc mixed data=meja;
class Clone MeJAConcen;
model PGConcen=Clone MeJAConcen;
random Clone;
lsmeans MeJAConcen/adj=tukey;
run;
proc sort data=meja out=meja2;
by MeJAConcen;
run;
proc means data=meja2 noprint;
by MeJAConcen;
var GAConcen PGConcen;
output out=meja2 mean=m1-m6 stderr=s1-s6;
run;
proc print;
run;
```

APPENDIX B. ANOVA ANALYSIS RESULTS

ANOVA of the effect of medium type and light condition on the fresh and dry weight of *E. ulmoides* hairy roots.

Weight	Effect	Num DF	Den DF	F Value	Pr>F
Fresh	Medium	2	20	100.17	<.0001
	lightcondition	1	20	4.42	0.0483
	Medium*lightconditio	2	20	0.06	0.9375
Dry	Medium	2	24	89.29	<.0001
	lightcondition	1	24	2.59	0.1208
	Medium*lightconditio	2	24	0.20	0.8236

ANOVA of the effect of initial pH on the fresh and dry weight of *E. ulmoides* hairy roots.

Weight	Effect	Num DF	Den DF	F Value	Pr> F
Fresh	pH	5	20	2.68	0.0517
Dry	pH	5	20	1.82	0.1553

ANOVA of the effect of medium strength on fresh and dry weight of *E. ulmoides* hairy roots.

Weight	Effect	Num DF	Den DF	F Value	Pr> F
Fresh	Strength	3	12	18.36	<.0001
Dry	Strength	3	12	45.66	<.0001

ANOVA of the effect of basal medium strength on fresh and dry weight of *E. ulmoides* hairy roots.

Weight	Effect	Num DF	Den DF	F Value	Pr> F
Fresh	Nutrient	2	24	6.81	0.0045
	Sucrose	2	24	68.60	<.0001
	Nutrient*Sucrose	4	24	2.74	0.0520
Dry	Nutrient	2	24	7.18	0.0036
	Sucrose	2	24	191.28	<.0001
	Nutrient*Sucrose	4	24	4.13	0.0110

ANOVA of the effect of the size of flask on the growth of *E. ulmoides* hairy roots.

Effect	Num DF	Den DF	F Value	Pr> F
Volume	3	6	2.55	0.1517

ANOVA of the auxin effect on the fresh and dry weight of *E. ulmoides* hairy roots.

Weight	Effect	Num DF	Den DF	F Value	Pr> F
Fresh	Auxin	6	21	13.34	<.0001
Dry	Auxin	6	21	27.57	<.0001

ANOVA for the effect of nutrient strength and sucrose concentration on the fresh and dry weight of *E.ulmoides* hairy roots.

Weight	Effect	Num DF	Den DF	F Value	Pr> F
Fresh	Nutrient	2	24	6.81	0.0045
	Sucrose	2	24	68.60	<.0001
	Nutrient*Sucrose	4	24	2.74	0.0520
Dry	Nutrient	2	24	7.18	0.0036
	Sucrose	2	24	191.28	<.0001
	Nutrient*Sucrose	4	24	4.13	0.0110

ANOVA of the effect of medium type and light condition on GA, PG and CA content of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	Medium	2	20	3.95	0.0359
	Light	1	20	3.44	0.0783
	Medium*Light	2	20	0.08	0.9219
PG	Medium	2	20	20.08	<.0001
	Light	1	20	163.01	<.0001
	Medium*Light	2	20	1.60	0.2272
CA	Medium	2	20	31.88	<.0001
	Light	1	20	102.98	<.0001
	Medium*Light	2	20	9.97	0.0010

ANOVA of the effect of medium type and light condition on GA, PG and CA yield of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	Medium	2	20	4.54	0.0236
	Light	1	20	2.96	0.1005
	Medium*Light	2	20	0.76	0.4801
PG	Medium	2	20	65.58	<.0001
	Light	1	20	18.02	0.0004
	Medium*Light	2	20	2.42	0.1145
CA	Medium	2	20	42.99	<.0001
	Light	1	20	35.64	<.0001
	Medium*Light	2	20	7.15	0.0045

ANOVA of the effect of initial pH on GA, PG and CA content of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	5	20	0.99	0.4492
PG	SampleName	5	20	3.19	0.0280
CA	SampleName	5	20	1.67	0.1887

ANOVA of fresh weight of different *E. ulmoides* hairy root lines.

Weight	Effect	Num DF	Den DF	F Value	Pr> F
Fresh	Line	7	16	23.87	<.0001
Dry	Line	7	16	17.41	<.0001

ANOVA of the effect of initial pH on GA, PG and CA yield of *E. ulmoides* hairy roots

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	5	20	1.01	0.4350
PG	SampleName	5	20	0.54	0.7426
CA	SampleName	5	20	2.03	0.1177

ANOVA of the effect of auxin on GA, PG and CA content of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	6	18	41.68	<.0001
PG	SampleName	6	18	156.11	<.0001
CA	SampleName	6	18	57.23	<.0001

ANOVA of the effect of auxin on GA, PG and CA yield of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	6	18	57.98	<.0001
PG	SampleName	6	18	156.11	<.0001
CA	SampleName	6	18	43.70	<.0001

ANOVA of the effect of medium strength on GA, PG and CA content of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	3	9	0.84	0.5071
PG	SampleName	3	9	10.24	0.0029
CA	SampleName	2	6	35.08	0.0005

ANOVA of the effect of medium strength on GA, PG and CA yield of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	3	9	8.30	0.0058
PG	SampleName	3	9	47.74	<.0001
CA	SampleName	2	6	34.12	0.0005

ANOVA of the effect of medium strength and sucrose concentration on GA, PG and CA content of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	Nutrient	2	24	1.84	0.1810
	sugar	2	24	0.78	0.4717
	Nutrient*sugar	4	24	1.45	0.2487
PG	Nutrient	2	24	28.10	<.0001
	sugar	2	24	18.31	<.0001
	Nutrient*sugar	4	24	1.58	0.2119
CA	pH	1	33	21.04	<.0001
	Sugar	1	33	12.29	0.0013
	pH*Sugar	1	33	1.11	0.3000
	Auxin	2	33	19.71	<.0001
	pH*Auxin	2	33	0.15	0.8579
	Auxin*Sugar	2	33	0.43	0.6510
	pH*Auxin*Sugar	2	33	1.55	0.2271

ANOVA of the effect of medium strength and sucrose concentration on GA, PG and CA yield of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	Nutrient	2	24	0.46	0.6349
	sugar	2	24	19.97	<.0001
	Nutrient*sugar	4	24	0.62	0.6525
PG	Nutrient	2	24	10.95	0.0004
	sugar	2	24	109.74	<.0001
	Nutrient*sugar	4	24	3.64	0.0187
CA	pH	1	33	15.38	0.0004
	Sugar	1	33	17.67	0.0002
	pH*Sugar	1	33	1.54	0.2234
	Auxin	2	33	6.41	0.0045
	pH*Auxin	2	33	0.01	0.9861
	Auxin*Sugar	2	33	0.37	0.6964
	pH*Auxin*Sugar	2	33	0.74	0.4850

ANOVA of the effect of flask size on GA,PG and CA content of *E. ulmoides* hairy roots

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	3	6	4.22	0.0633
PG	SampleName	3	6	0.55	0.6684
CA	SampleName	3	6	19.30	0.0017

ANOVA of the GA, PG and CA content in different hairy root lines.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	7	16	4.53	0.0059
PG	SampleName	7	16	14.31	<.0001
CA	SampleName	7	16	14.34	<.0001

ANOVA of the GA, PG and CA yield in different hairy root lines.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	7	16	7.31	0.0005
PG	SampleName	7	16	4.25	0.0078
CA	SampleName	7	16	2.34	0.0753

ANOVA of the effect of pH, auxin type, and sucrose concentration on GA yield of *E. ulmoides* hairy roots.

Effect	Num DF	Den DF	F Value	Pr> F
pH	1	33	6.12	0.0186
Sugar	1	33	27.15	<.0001
pH*Sugar	1	33	0.06	0.8028
Auxin	2	33	25.85	<.0001
pH*Auxin	2	33	2.60	0.0892
Auxin*Sugar	2	33	4.21	0.0236
pH*Auxin*Sugar	2	33	2.00	0.1509

ANOVA of the effect of basal medium strength and sucrose concentration on PG yield of *E.ulmoides* hairy roots.

Effect	Num DF	Den DF	F Value	Pr> F
Nutrient	2	24	10.95	0.0004
Sugar	2	24	109.74	<.0001
Nutrient*sugar	4	24	3.64	0.0187

ANOVA of GA, PG and CA content in growth period of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	Week	5	15	3.75	0.0211
PG	Week	5	15	2.89	0.0504
CA	Week	4	12	8.61	0.0016

ANOVA of GA yield in growth period of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	Week	5	15	17.67	<.0001
PG	Week	5	15	80.98	<.0001
CA	Week	4	12	13.49	0.0002

ANOVA of the effect of MeJA on GA content of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	MeJAConcen	5	15	2.06	0.1285
PG	MeJAConcen	5	15	5.88	0.0033
CA	MeJAConcen	5	15	19.35	<.0001

ANOVA of SA on GA content of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	5	14	11.57	0.0001
PG	SampleName	5	14	73.79	<.0001
CA	SampleName	5	14	23.78	<.0001

ANOVA of the effect of light elicitation on GA, PG and CA content of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	2	4	4.45	0.0963
PG	SampleName	2	4	0.06	0.9385
CA	SampleName	2	4	5.40	0.0730

ANOVA of the effect of light elicitation on GA, PG and CA yield of *E. ulmoides* hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	2	4	4.52	0.0942
PG	SampleName	2	4	5.12	0.0788
CA	SampleName	2	4	18.41	0.0096

ANOVA for the GA content in different sample source of *E. ulmoides*.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	SampleName	4	10	3909.00	<.0001
PG	SampleName	4	10	1792.97	<.0001
CA	SampleName	4	10	169.26	<.0001

ANOVA of comparison of GA, PG and CA content between normal and VHb hairy roots.

Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	Source	1	16	0.51	0.4874
PG	Source	1	16	0.08	0.7865
CA	Source	1	16	17.82	0.0006

ANOVA of comparison of GA, PG and CA yield between normal and VHb hairy roots.

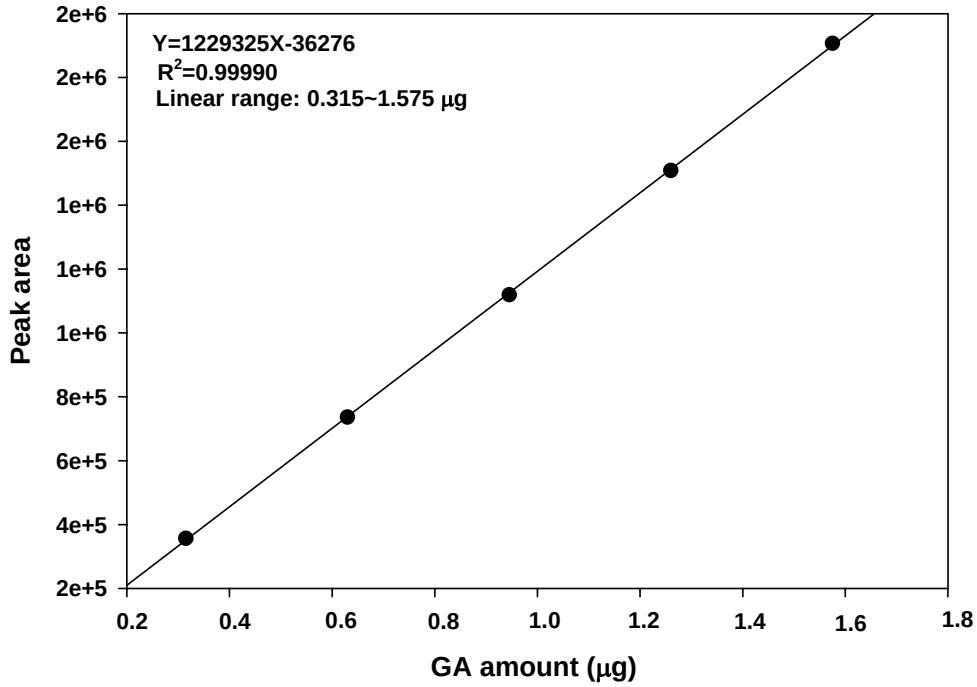
Compound	Effect	Num DF	Den DF	F Value	Pr> F
GA	Source	1	16	5.31	0.0350
PG	Source	1	16	21.47	0.0003
CA	Source	1	16	24.60	0.0001

ANOVA of the fresh and dry weight comparison between normal and VHb hairy roots in four weeks.

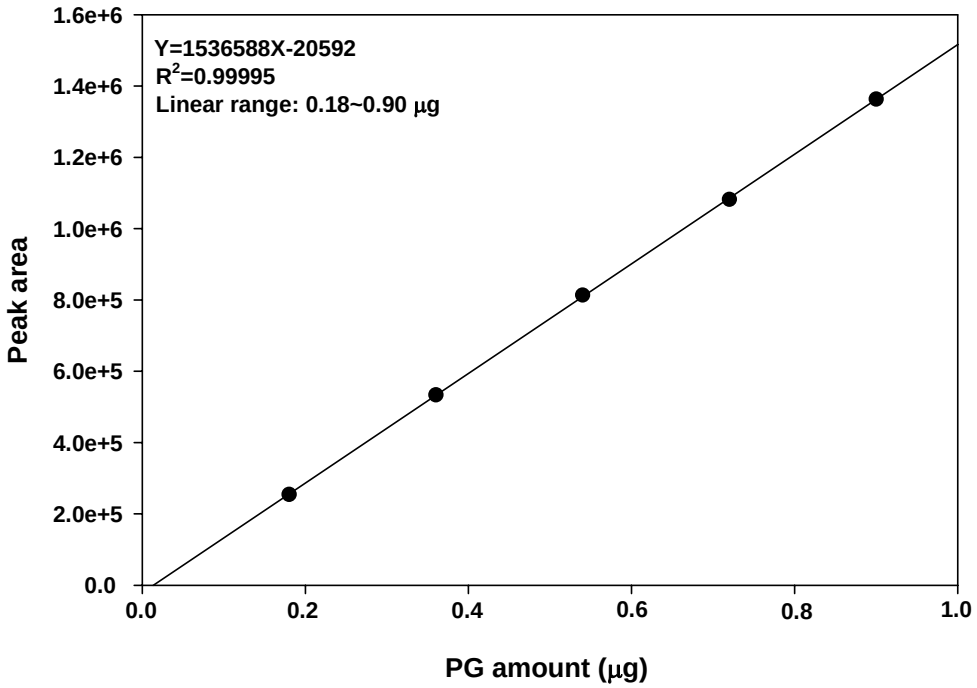
Weight	Effect	Num DF	Den DF	F Value	Pr> F
Fresh	Source	1	16	21.75	0.0003
Dry	Source	1	16	10.49	0.0051

APPENDIX C. STANDARD CURVES FOR HPLC ANALYSIS

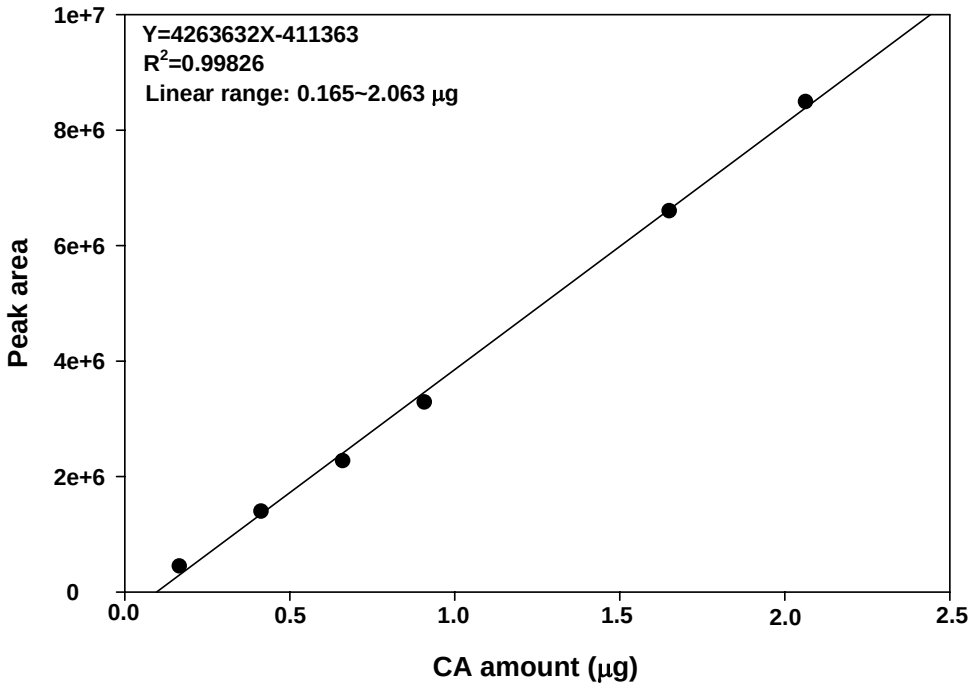
Calibration curve of GA performed on HPLC



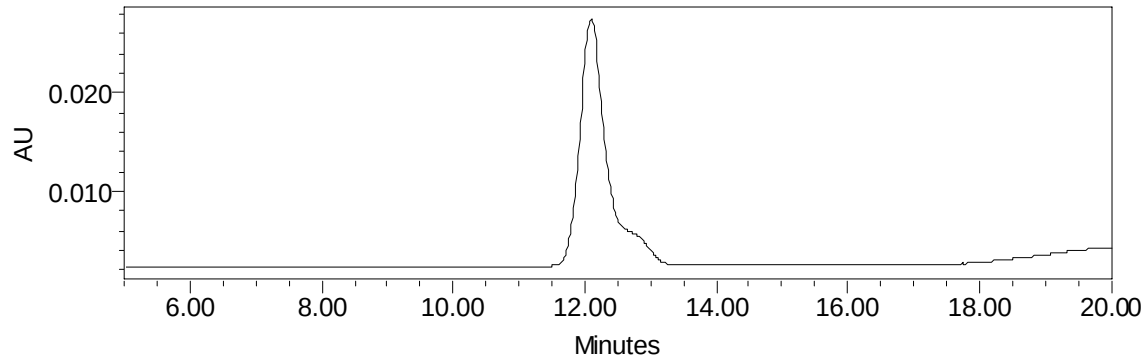
Calibration curve of PG performed on HPLC



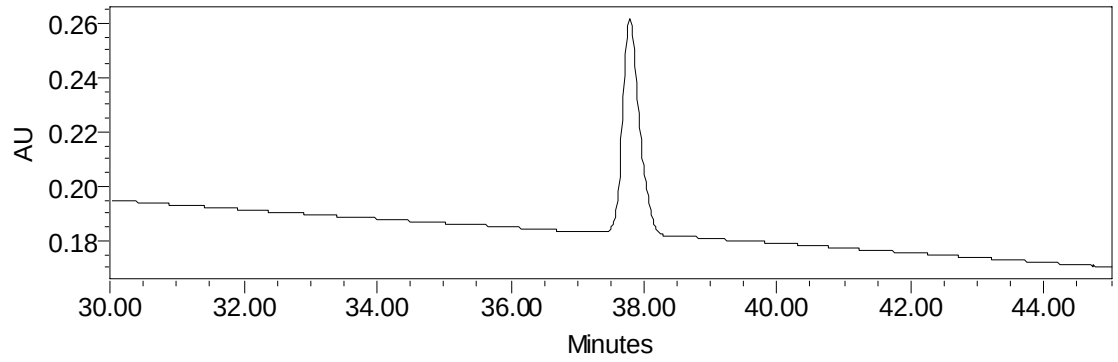
Calibration curve of CA performed on HPLC



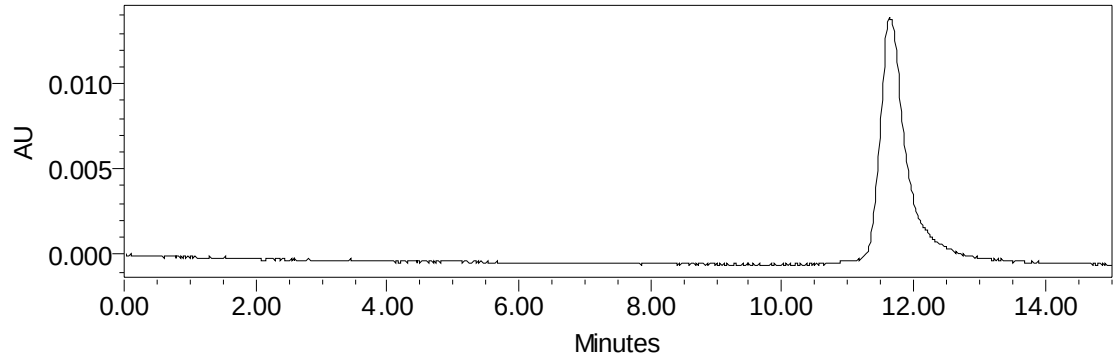
Chromatogram of GA



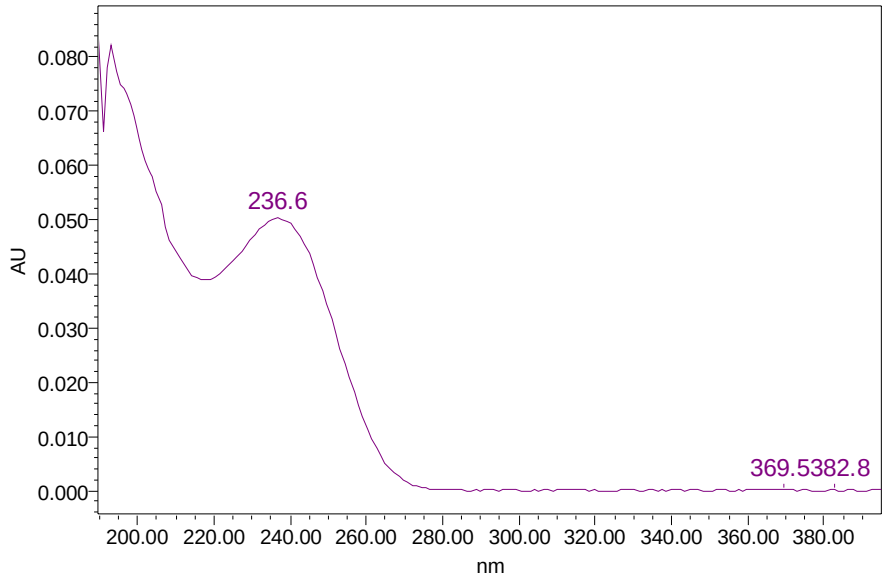
Chromatogram of PG



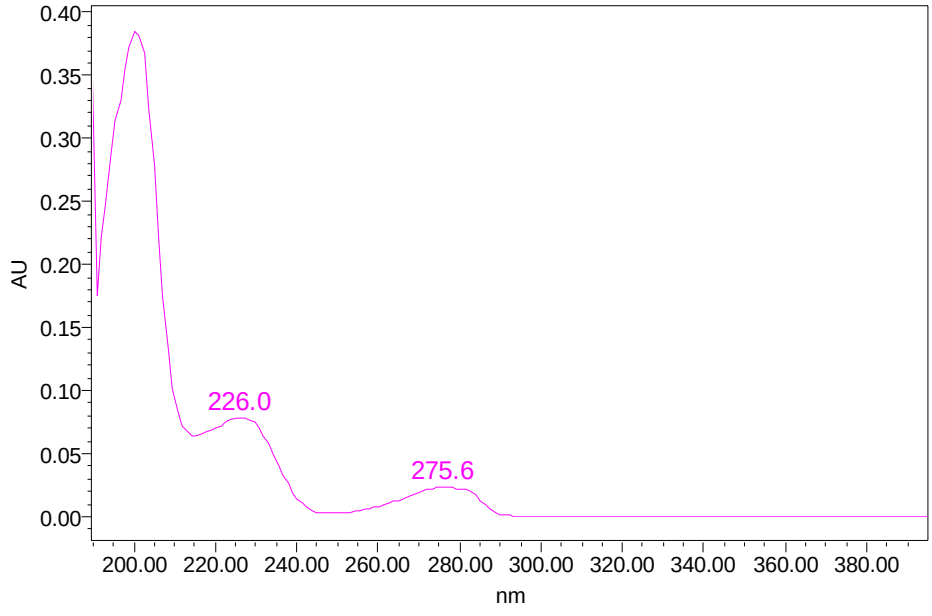
Chromatogram of CA



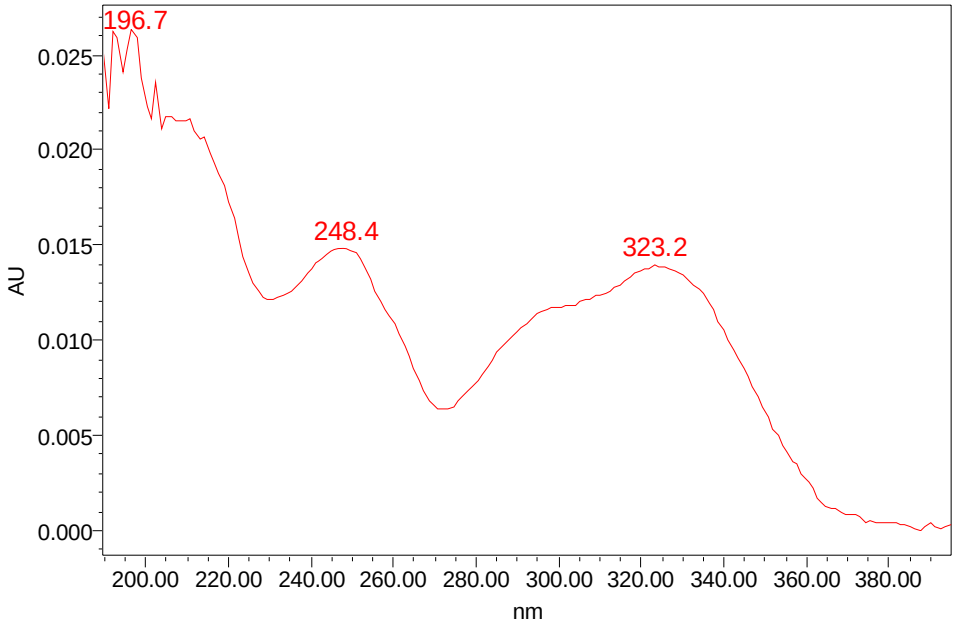
UV absorbance spectrum of GA



UV absorbance spectrum of PG



UV absorbance spectrum of CA



APPENDIX D. MEDIUM COMPOSITION

B5 medium

Component	Concentration (mg/L)
Ammonium Sulfate	134
Boric Acid	3
Calcium Chloride, Anhydrous	113.24
Cobalt Chloride•$6H_2O$	0.025
Cupric Sulfate•$5H_2O$	0.025
Na_2EDTA	37.26
Ferrous Sulfate• $7H_2O$	27.8
Magnesium Sulfate	122.09
Manganese Sulfate•H_2O	10
Molybdic Acid (Sodium Salt) •$2H_2O$	0.25
Potassium Iodide	0.75
Potassium Nitrate	2500
Sodium Phosphate Monobasic	150
Zinc Sulfate• $7H_2O$	2
myo-Inositol	100
Nicotinic Acid (Free Acid)	1
Pyridoxine•HCl	1
Thiamine•HCl	10

MS medium

Component	Concentration (mg/L)
Ammonium Nitrate	1650
Boric Acid	6.2
Calcium Chloride, Anhydrous	332.2
Cobalt Chloride•$6H_2O$	0.025
Cupric Sulfate•$5H_2O$	0.025
Na_2EDTA	37.26
Ferrous Sulfate•$7H_2O$	27.8
Magnesium Sulfate	180.7
Manganese Sulfate•H_2O	16.9
Molybdic Acid (Sodium Salt) •$2H_2O$	0.25
Potassium Iodide	0.83
Potassium Nitrate	1900
Potassium Phosphate, Monobasic	170
Zinc Sulfate. •$7H_2O$	8.6
Glycine	2
myo-Inositol	100
Nicotinic Acid (Free Acid)	0.5
Pyridoxine•HCl	0.5
Thiamine•HCl	0.1

WPM medium

Component	Concentration (mg/L)
Ammonium Nitrate	400
Boric Acid	6.2
Calcium Chloride, Anhydrous	72.5
Calcium Nitrate	386
Cupric Sulfate•$5H_2O$	0.25
Na_2EDTA	37.3
Ferrous Sulfate•$7H_2O$	27.85
Magnesium Sulfate	180.7
Manganese Sulfate•H_2O	22.3
Molybdic Acid(Sodium Salt) •$2H_2O$	0.25
Potassium Phosphate, Monobasic	170
Potassium Sulfate	990
Zinc Sulfate•$7H_2O$	8.6
Glycine (Free Base)	2
myo-Inositol	100
Nicotinic Acid (Free Acid)	0.5
Pyridoxine•HCl	0.5
Thiamine•HCl	1

APPENDIX E. ABBREVIATIONS

ANOVA: analysis of variance

B5: Gamborg and Eveleigh medium

CA: chlorogenic acid

CTAB: acetyl trimethyl ammonium bromide

dw: dry weight

GA: geniposidic acid

HPLC: high pressure liquid chromotagraphy

MeJA: metyl jasmonic acid

MeOH: methanol

MS: Murashige and Skoog medium

OD: optical density

ORF: open reading frame

PCR: polymerase chain reaction

PG: pinoresinol diglucoside

SA: salicylic acid

VHb: Bitreoscilla hemoglobin

WPM: Lloyd and McCrown medium

YMB: yeast mannitol broth

VITA

Xiaojun Wu was born on February 7, 1976, in Rugao, Jiangsu Province of People's Republic of China. In 1993, he entered Nanjing University and got his Bachelor of Science degree majoring in biology from Department of Biology of the university four years later. He continued his graduate study in Institute of Chinese Materia Medica of Shanghai University of Traditional Chinese Medicine majoring in bioengineering of medicinal plants and earned the degree of Master of Science there in 2000.

In the following three years, he worked in Institute of Chinese Materia Medica as Research Associate. His research there included molecular biology, drug screening through various cell and animal models, and pharmacology and toxicity studies. He participated in the research of many projects sponsored by various governmental agencies of China, including the State Science and Technology Commission, the State Administration of Traditional Chinese Medicine and the National Nature Science Foundation.

In 2003, he enrolled in Louisiana State University and pursued his doctoral degree at the School of Renewable Natural Resources majoring in biotechnology of medical plants. In May 2007, he was awarded the degree.

Xiaojun is an active member of American Society of Pharmacognosy and Honor Society of Phi Kappa Phi. He is interested in research and development of botanical medicine, molecular biology study, and various cell or animal models for drug screening.