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Effect of Gibberellic Acid and Chlormequat Chloride on Growth, Coumarin Content and Root Yield of *Angelica dahurica* var. Formosana

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ABSTRACT

The effects of the plant growth promoter gibberellic acid (GA_3) and plant growth retardant chlormequat chloride (CCC) on biomass and quality of *Angelica dahurica* var. formosana plants were investigated employing physiological and biochemical methods. Plants were sprayed with GA_3 or CCC at rosette stage and coumarin content was analyzed by high performance liquid chromatography (HPLC) after harvest. Two coumarins in root were detected: isoimperatorin and imperatorin. The root content of imperatorin was not affected by application either GA_3 or CCC, while isoimperatorin was increased to 127% after treatment with CCC (400 mg L^{-1}). The lowest concentrations of GA_3 (50 mg L^{-1}) and CCC (400 mg L^{-1}) increased root yield to 157 and 139%, respectively. We note here that GA_3 and CCC might be used in improving the yield of *A. dahurica* var. formosana and maintaining the coumarin content.

Keywords: *Angelica dahurica* var. formosana, Plant growth promoter, Biomass, Coumarin content.

INTRODUCTION

The root of *Angelicae dahurica* var. formosana is traditionally considered as one of the most common components of Traditional Chinese Medicine (TCM) prescriptions which are widely used in China (State Pharmacopoeia Commission of PRC, 2005). Generally, *A. dahurica* var. formosana is sown in early October and harvested the next July. However, some plants may display a premature bolting and flowering before harvest, making the root lignified and the content of coumarins of these plants are significantly lower than non-bolting plants. Thus, controlling the early bolting is very important for the root production of *A. dahurica* var. formosana.

It is believed that coumarins are responsible for part of the pharmaceutical properties of *A. dahurica* var. formosana. Three major coumarins including imperatorin, oxypeucedanin, and isoimperatorin and many other coumarins have been isolated from the root of *A. dahurica* var. formosana (Wei and Ito, 2006). Among these, imperatorin and isoimperatorin are especially known for their pharmacological activity and often used as reference standards in the quality control of *A. dahurica* var. formosana and its products (State Pharmacopoeia Commission of PRC, 2005). What is more, previous studies have suggested that the imperatorin strongly suppresses cyclin D1 expression and arrests the cells at the G1 phase of the cell cycle, which highlights that it might have a potential therapeutic role in the management of acquired immunodeficiency syndrome (AIDS) (Sancho *et al*., 2004).

Coumarins are found in higher plants where they originate from the phenylpropanoid

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pathway (Harborne, 1999). Most plants accumulating coumarins possess a highly inducible biosynthetic pathway, which can be triggered by various biotic (Hagemeier *et al*., 1999; Hamerski and Matern, 1988) and abiotic stresses (Eckey-Kaltenbach *et al*., 1994; Katz *et al*., 1998). Interestingly, previous research found inhibitory effects of coumarins on seed germination and root growth in radish plants (Aliotta *et al*., 1993), implicating complex effects of coumarins on plant growth and development (Svensson, 2006).

It is well known that GA_3 promotes plant growth and its secondary metabolite production (Jones, 2009). Exogenously applied GA_3 (0.5 mg L^{-1}) enhanced growth and promoted the accumulation of coumarin content in hairy root cultures of *Cichorium intybus* L. *cv.* Lucknow Local (Bais, 2001). However, some conflicting reports showed that GA_3 was ineffective or had slight effects on plant growth. By common literature consensus, the effects of spraying with GA_3 might depend on the particular species and surrounding factors (Hedden and Thomas, 2012). While CCC, as an inhibitor of gibberellin biosynthesis, has a profound effect on plant growth (Emam and Moaied, 2000), it not only retards stem growth, but also reverses root growth capacity. For example, GA_3 production in *Fusarium moniliforme* (*Gibberella fujikuroi*) is suppressed by adding CCC to the culture medium (Ninnemann *et al*., 1964), and CCC suppresses excessive vegetative growth, favors quality attributes and does not alter yield of cassava (*Manihot esculenta* Crantz cv. Rocha) (Medina *et al.*, 2012).

Although *A. dahurica* var. formosana is a popular herb used widely in China, relatively little progress has been made to increase root yield. One of the unanswered questions regarding *A. dahurica* var. formosana production is the effect of plant growth regulators on the organ development and yield formation of this plant, especially on controlling the premature bolting, which is crucial to the root yield. In this paper, we describe an experiment in which GA_3 and CCC were applied to *A. dahurica* var. formosana, in an attempt to enhance the plant growth and root yield as well as improvement of the coumarin content, specifically imperatorin and isoimperatorin, of this species.

MATERIALS AND METHODS

Plant Material, Experimental Design, and Treatments

The study was carried out at the second terrace of the Qingyi River in Yaan, China (Latitude: 29° 58' 60 N, Longitude: 103° 4' 60 E, Altitude: 580 m from sea level), within the marginal area of Sichuan Basin. The area has a climate zone characterized by a humid mild subtropical climate, with mean annual precipitation of 1774.3 mm, monthly mean temperatures of 25.3℃ for July and 6.1℃ for January, and mean annual temperature of 16.2℃.

The seeds of *Angelica dahurica* var. formosana were collected from Suining of Sichuan province in China and the plants were grown in the soil. The soil in the study area is purplish loam soil, formed on a deposit with weathering of purplish shale. Each experimental plot was 8 m^2 with 5 surrounding guard rows and the row spacing of 33 cm, while the plant spacing was 20 cm. The distance between the double plots nearby was 0.8 m; each plot comprised one replication of each treatment.

The treatments were different concentrations of GA_3 (50-, 100-, and 150 mg·L^{-1}) and CCC (400, 700, and 1000 mg L^{-1}) applied at the rate of 500 L ha^{-1}. Plants sprayed with distilled water were considered as control. Five-leaf stage shoots (180 days after sowing) were selected for spraying. During application, care was taken in order to avoid over-spraying nearby plots of different treatments.

The plant height (measured from the soil surface to the top of the highest leaf), the early bolting rate, the yield of the root and chlorophyll content expressed as SPAD (Soil

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Plant Analysis Development), and Chlorophyll Meter Reading (SCMR) of *A. dahurica* var. formosana were measured on 24 July at harvest. The SPAD meter can measure plant chlorophyll density *in vivo*, and it is considered as an ideal instrument for collecting data on leaf chlorophyll in the field without any destructive sampling, as also reported for cotton (Qu *et al.*, 2007) and rice (Asai *et al.*, 2009).

Plant chlorophyll content of different treatments was obtained through SCMR (Kashiwagi *et al*., 2006). The leaves from the same location of different individuals in each treatment were used to measure SCMR at harvest period. Five readings using the SPAD chlorophyll meter (Minolta SPAD-502 meter, Tokyo, Japan) were recorded on each leaflet beside the mid-rib. In recording the SCMR, care was taken to ensure that the SPAD meter sensor fully covered the leaf lamina avoiding interference from veins and midribs.

Samplings

Sampling event took place at harvest. Preparation of the crude extract was carried out according to the literature data (State Pharmacopoeia Commission of PRC, 2005). The dried roots of *A. dahurica* var. formosana were ground to powder (about 60 meshes). Established sample preparation method by ultrasonic extraction was followed: dried powder were crushed and extracted by 50 ml methanol (extraction solvent) at 40ºC and 40 kHz for 60 minutes. The extracts were then filtered through a 0.45 µm membrane filter to get rid of impurities before use. A volume of 10 µL was injected into the HPLC system for analysis.

Analytical Techniques

The HPLC analysis was performed according to the method in Chinese pharmacopoeia 2005 edition with an Agilent 300 Extend-C_{18} (5 µm, 250×4.6 mm) operated at 30ºC. The mobile phase was methanol: water (70:30, v/v). The effluent was monitored at 254 nm and the flow rate was kept at 1.0 ml min^{-1} constantly. The analyses were performed using an Agilent series 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with a quaternary pump, a diode array detector (DAD), an auto sampler, and a column compartment. The HPLC chromatogram is shown in Figure1.

Chemicals and Reagents

HPLC grade methanol was purchased from Burdick & Jackson Fish Scientific (Muskegon, MI, USA), imperatorin and isoimperatorin were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Ultra-pure water was generated with a Millipore Ultra-pure Water System (Milli-QRG, American Millipore Company, Bedford, MA, USA). GA_3 and CCC were purchased from Shanghai beitabiotech. Co., Ltd (Shanghai, China).

Statistical Analysis

The experiments were arranged as a randomized complete design with three replications. All samples were analyzed twice. Data derived were analyzed using one-way ANOVA followed by comparison of multiple treatment levels with control, using Fisher's *LSD* (Least Significant Difference) test.

RESULTS

Effects of GA_3 and CCC on Growth and Root Yield

GA_3 foliar spraying of *A. dahurica* var. formosana plants increased plant height, while CCC treatment showed opposite effect

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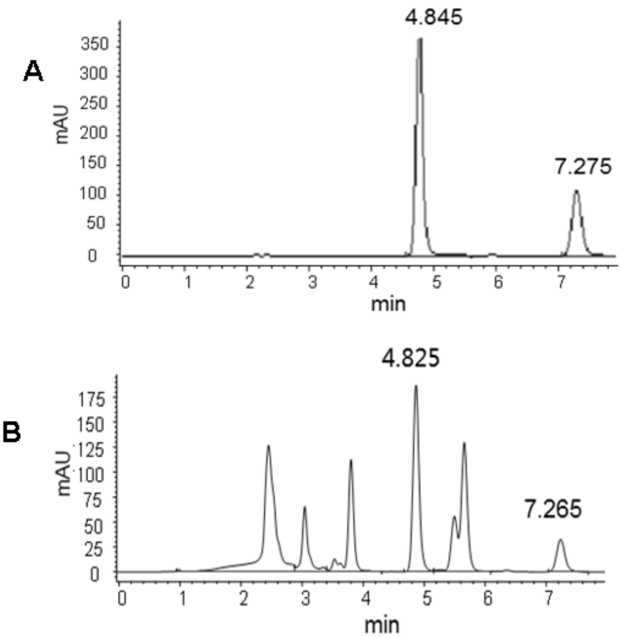

Figure 1. (A) HPLC chromatogram of standards of imperatorin (4.845) and isoimperatorin (7.275), (B) HPLC chromatogram of the root samples of *A. dahurica* var. formosana plants: imperatorin (4.825) and isoimperatorin (7.265), the other peaks were not characterized.

in a concentration dependent manner (Table 1).

SCMR is an index of chlorophyll content. The SCMR values of plants sprayed with GA_3 were all significantly lower than those sprayed with CCC and the control, except for the lowest concentration of 50 mg L^{-1} GA_3 (Table 1). SCMR of the plants was directly proportional to the spraying concentration of CCC, and this value was significantly affected only in the case of plants sprayed with 1,000 mg L^{-1} CCC (Table 1).

Plants sprayed with GA_3 (50 mg L^{-1}) displayed lower (17.3%) bolting rate compared with the control group (23.4%), while the higher concentrations of GA_3 accelerated bolting (Table 1). The application of CCC significantly ($P< 0.05$) reduced bolting ratio at all concentration

Table 1. Effects of gibberelic acid (GA_3) and chlormequat (CCC) ($mg·L^{-1}$) on plant height (cm), chlorophyll content expressed as SPAD meter readings (SCMR), stem diameter (cm), bolting rate (%), and root yield ($kg·FW^{-1}$) of *A. dahurica* var. formosana plants. [a]

Treatments	Concentration ($mg·L^{-1}$)	Plant height (cm)	SCMR	Bolting rate (%)	Root yield ($kg·FW^{-1}$)
CCC	400	66.60±12.38 d	34.80±3.86 bc	12.21±1.52 e	32.30±2.25a
CCC	700	60.70±11.93 de	38.50±4.93 ab	8.09±1.71 ef	28.30±2.18 ab
CCC	1000	53.5±9.86 e	40.15±4.69 a	4.90±1.29 f	19.30±1.85 c
Control	0	82.50±13.65 c	34.95±4.84 bc	23.75±1.75 c	23.30±2.18 bc
GA_3	50	95.40±13.83 b	31.05±4.59 cd	17.29±1.62 d	36.60±3.25 a
GA_3	100	106.00±25.26 a	29.95±3.79 d	28.01±1.85 b	20.60±2.17 bc
GA_3	150	111.85±11.89 a	29.35±3.52 d	41.31±2.26 a	19.30±1.65 c

[a] Values are means ± S.E. (n= 3). Means marked with different letters are significantly different at $p < 0.05$ (LSD test). FW-fresh weight; bolting rate (%)- number of premature bolting plants/whole group plants.

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(Table 1).

The root yield of the plants significantly (P< 0.05) increased in the lowest concentration of GA_3 (50 mgL^{-1}) or CCC (400 mg L^{-1}) applied, while the higher concentrations of these plant growth regulators reduced it (Table 1).

Effects of GA_3 and CCC on the Coumarin Content

Coumarin content in *A. dahurica* var. formosana plants was not affected in almost all treatments (GA_3 or CCC), except for the lowest concentration (400 mg L^{-1}) of CCC. Furthermore, at this lowest concentration of CCC, the accumulation of isoimperatorin increased to 127% as compared to the control plants (Table 2). Imperatorin content did not change, regardless of the treatment.

DISCUSSION

Gibberellins are one of the main regulators of plant growth and development (Hooley, 1994), and they normally act by signaling the removal of proteins that repress growth, thus promoting cell proliferation and elongation (Olszewski *et al*., 2002; Ubeda-Tomas *et al*., 2009). Exogenously applied gibberrelic acid (GA_3) increases plant height and, subsequently, enhances dry weight (Menzel, 1980; Sharma *et al*., 1999). In this study, higher concentrations of GA_3 resulted in significant increase of the plant height (Table 1) and generally induced premature bolting, except for the lowest concentration. Moreover, CCC, as a GA_3 biosynthesis inhibitor, reduced plant height and, thus, produced an effect on root growth performance (Table 1) as it is expected for growth retardant (Rademacher, 2000). In this work, CCC treatments postponed premature bolting and increased root yield (Table 1), which is in the line with previously reported data for Alaska pea (Tanimoto, 1988).

Understanding how roots grow and how hormones control their growth is crucial for improving the root yield. The effects of exogenous application of GA_3 and CCC on root yield and its coumarin content of *A. dahurica* var. formosana plant showed that the lowest concentrations of GA_3 (50 mg L^{-1}) and CCC (400 mg L^{-1}) increased the root yield. These data indicated that there may be a natural hormone balance in this plant, therefore, only a slight change of their concentrations might be beneficial for the plant growth and development. Although it is well known that the hormonal balance has a direct influence on the plant growth (Aarrouf *et al*., 1999), the hormonal mechanisms that drive root morphogenesis in this plant, still remain elusive. Further research is needed to delineate the complex mechanism(s) of hormonal impact on root growth and development.

Coumarin content, specifically, imperatorin and isoimperatorin, is one of the

Table 2. Effect of gibberrelic acid (GA_3) and chlormequat (CCC) (mg L^{-1}) on imperatorin and isoimperatorin content in the root (% of dry root powder) of *A. dahurica* var. formosana plants [a].

Treatments	Concentration (mg L^{-1})	Isoimperatorin (%)	Imperatorin (%)
CCC	400	0.20±0.015 a	0.33±0.033 a
CCC	700	0.14±0.0097 b	0.35±0.046 a
CCC	1000	0.10±0.0084 bc	0.25±0.028 a
Control	0	0.10±0.0067 c	0.31±0.035 a
GA_3	50	0.10±0.0071 bc	0.28±0.026 a
GA_3	100	0.11±0.0086 bc	0.30±0.035 a
GA_3	150	0.10±0.0072 bc	0.29±0.033 a

[a] Values are means±SE (n= 3). Means marked with different letters are significantly different at *P*< 0.05 (LSD test).

most important criteria for determining the quality of *A. dahurica* var. formosana plant (State Pharmacopoeia Commission of PRC, 2005), therefore, maintaining or increasing the accumulation of coumarin is an effective way to guarantee the crude drug quality. To the best of our knowledge, this is the first study on the effects of GA_3 and CCC on imperatorin and isoimperatorin content in this plant. It has been previously reported that there is a difference in the metabolic pathways of the bioconversion of isoimperatorin and imperatorin. Thus, they differ in the position of prenyloxy side-chain combined to C-5 or C-8 at furanocoumarin skeleton (Marumoto, 2009). Therefore, it is reasonable to expect diverse effects of GA_3 and CCC on accumulation of isoimperatorin and imperatorin. None of GA_3 concentrations applied affected coumarin root content (Table 2). Moreover, CCC (400 or 700 mg L^{-1}) displayed significant ($P< 0.05$) effect on isoimperatorin, while the highest concentration CCC (1,000 mg L^{-1}) slightly inhibited the accumulation of imperatorin (Table 2). Environmental conditions affecting the growth of plants may also influence coumarin synthesis and accumulation in *A. dahurica* var. formosana plants. The yield and quality of this plant were found to be affected by lots of factors, such as date of seeding, fertilization, continuous cropping, and planting density, as previously reported by Chen and Ding (1999). For instance, in onion plants, CCC and GA_3 were reported to improve the growth and yield, depending on the spraying time (Karim *et al*., 2002).

Besides the effects of GA_3 and CCC on plant growth, chlorophyll content was also affected by these treatments in a concentration-dependent manner. Chlorophyll is an indicator of crop health and productivity (Ruttanaprasert *et al.*, 2012). These multiple effects of GA_3 and CCC on plant growth and development have been previously reported (Harberd, 1998; Passam, 2008). In this work, chlorophyll contents of the plants sprayed with GA_3 were all significantly lower ($P< 0.05$) than those sprayed with CCC (Table 1). A higher chlorophyll content of leaves and reduced early bolting rate might result in efficient transport of sucrose from the leaves to the roots, leading to higher root yield, due to more carbon availability for the sink organs. In contrast to GA_3, the stem diameter and plant height were reduced by CCC treatment in a concentration-dependent manner (Table 1). The application of GA_3 and CCC on different species displayed great diversity in their effects. Thus, in *Taxus* plants treated with GA_3 (33 mg L^{-1}) or CCC (400 mg L^{-1}), the root growth was decreased, while 100 mg·L^{-1} CCC exhibited promotive effect on root growth (Wickremesinhe *et al*., 1996). In contrast to *Lemna minor,* root growth was not affected by the addition of GA_3 to the culture medium (Inadal *et al*., 2000).

In conclusion, CCC and GA_3 play important roles in the root yield and quality of *A. dahurica* var. formosana plants. Some of the treatments enhanced plant productivity resulting in higher total yield. Nevertheless, it must be noted that the positive action of PGRs depends on the concentration, PGRs type, and even the environmental conditions before, during, and after application, etc. Therefore, further research using different cultivars is needed to generalize the effect of such growth stimulating substances on *A. dahurica* var. formosana yield and quality. GA_3 (50 mg L^{-1}) and CCC (400 mg L^{-1}) could increase root yield and maintain the coumarin content of this plant.

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اثر جیبرلیک اسید و کلرید کلرمیکوات روی رشد ، محتوی کومارین و عملکرد ریشه گیاه سنبل خطایی *Angelica dahurica* var. Formosana

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چکیده

در این پژوهش، با کار برد روش های فیزیولوژیکی و بیو شیمیایی، اثرات جیبرلیک اسید(GA_3) به عنوان ماده افزاینده رشد گیاه و کلرید کلرمیکوات(CCC) به عنوان ماده بازدارنده رشد، روی زیست توده و کیفیت گیاه سنبل خطایی*Angelica dahurica* var. Formosana بررسی شد. جیبرلیک اسید یا ماده کلرید کلرمیکوات در مرحله وردمانی (rosette) روی گیاهان پاشیده شد و محتوی کومارین آنها بعد از برداشت، با کار برد طیف سنجی مایع دقیق (HPLC) تجزیه گردید. دو کومارین در ریشه آنها شناسایی شد:isoimperatorin وimperatorin. مقدار imperatorin ریشه تحت تاثیر GA3یا CCCقرار نگرفت در حالی که isoimperatorin در اثر افزایش 400 میلی گرم در لیتر CCC به مقدار ۱۲۷٪ افزایش یافت. کمترین غلظت جیبرالیک اسید(۵۰ میلی گرم در لیتر) و CCC(۴۰۰میلی گرم در لیتر) تولید ریشه را به ترتیب ۱۵۷٪ و ۱۳۹٪ افزایش داد. نتایج چنین اشاره دارد که می توان GA3 وCCC را برای بهبود تولید گیاه *A. dahurica var. formosana* و حفظ محتوی کومارین به کار برد.

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