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Original Research Article

Improved production of chlorogenic acid from cell suspension cultures of *Lonicera macranthoids*

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Abstract

Purpose: *To evaluate the potential of Lonicera macranthoides Hand. -Mazz. Yulei1 suspension culture system for enhanced production of the main secondary metabolite, chlorogenic acid.*
Methods: *The callus of L. macranthoides Hand.-Mazz. "Yulei1" was suspension cultured in B5 liquid medium supplemented with different plant growth regulators. Biomass accumulation was calculated by weight method and chlorogenic acid production was measured using high performance liquid chromatography (HPLC). HPLC was carried out on C18 analytical column at 35 °C and the detection wavelength was set at 324 nm.*
Results: *The results showed that maximum accumulation of biomass and chlorogenic acid were achieved 15 days after culture growth. The optimized conditions for biomass accumulation and chlorogenic acid production were 50 g/L of inoculum on fresh weight basis, B5 medium supplemented with plant growth regulators, 30 - 40 g/L sucrose and initial medium pH of 5.5. Maximum accumulation of chlorogenic acid and biomass were observed when the culture medium was supplemented with 2.0 mg/L6-BA. Optimal accumulation of chlorogenic acid was observed using combination of hormones 2.0 mg/L 6-Benzyladenine (BA) + 0.5 mg/L2, 4-Dichlorophenoxyacetic acid (2,4-D), while optimal accumulation of biomass was observed with 2.0 mg/L 6-BA + 2.0 mg/L2, 4-D. In addition, phenylalanine also contributed to the synthesis of chlorogenic acid at a concentration > 50 mg/L.*
Conclusion: *Cell suspension cultures of L. macranthoides Hand.-Mazz. "Yulei1" have successfully been established. The findings provide a potential basis for large scale production of chlorogenic acid using cell suspension cultures of L. macranthoides.*

Keywords: *Lonicera macranthoides, Cell suspension culture, Chlorogenic acid, Phenylalanine, Optimization*

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INTRODUCTION

Lonicera is a pharmacologically important medicinal plant and have long been used in Chinese traditional medicines. It is used in nearly one third of Chinese traditional prescriptions [1, 2]. *Lonicera macranthoides* Hand.-Mazz. "Yulei1" was approved as a new variety of honeysuckle with species number S-SV-LM-005-2008, and also authorized as the first medicinal plant variety in Chongqing in 2008 [3]. Compared with traditional honeysuckle, this variety has high yield and stress resistance, and most importantly high content of chlorogenic acid (5.16 - 7.37 %) [3].

It is well known that the level of chlorogenic acid is the main evaluation index of quality of honeysuckle [4]. Chlorogenic acid is a free radical scavenger, antibacterial, anti-inflammatory, antiviral, hypoglycemic, and in addition to this it also inhabits oxidation of lipids

and protects liver and gallbladder [5-8]. The anti-cancer and anti-HIV properties of chlorogenic acid have also been documented in the recent years [9]. As an antioxidant, chlorogenic acid has a wide range of applications in medicine, food and other fields. Despite the fact that the production of chlorogenic acid is increasing rapidly in China, still it does not meet the requirement of enormously growing population.

Traditional extraction of chlorogenic acid consumes a great amount of the raw materials of honeysuckle foliage. Plant cell culture is an attractive alternative technology for enhancing production of secondary metabolites [10]. More than 400 species of medicinal plants have been studied through cell culture, and over 600 secondary metabolic products are isolated from cultured cells. It is shown that more than 60 medicinal plants have higher content of metabolites in culture cells than in original species [11]. In particular, plant cell suspension cultures containing undifferentiated cells are attractive due to their relative similarity to microbial cell culture systems [12]. *L. macranthoides* Hand.-Mazz. "Yulei1" is a new species and its cell suspension culture system has not been established yet. This study was designed to establish cell suspension culture system and investigate the production of chlorogenic acid in the suspension cultures of *L. macranthoides* Hand.-Mazz.

EXPERIMENTAL

Plant material and establishment of cell suspension culture

Leaf explants were obtained from the young leaves of *L. macranthoides*. They were briefly washed with running tap water and surface sterilized with 75 % ethanol for 30 s and then 0.1 % mercuric chloride for 1 - 4 min. After sterilization, they were washed 4 times with sterile distilled water. Under sterile conditions, leaf explants were cut into the size of 0.5 cm in diameter and then inoculated onto full strength vit B5 medium (0.7 % agar, w/v) [13] supplemented with 30 g/L sucrose, 2.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L 6-benzylaminopurine (6-BA). Cultures were incubated in the growth chambers at 25 ^{o}C with a 16 h photoperiod (40 µmol m^{-2} s^{-1}) provided by 40W white fluorescent lamps.

The callus started to develop after 21 days and eventually used for cell suspension cultures after subculture for three times. Cell suspension cultures were initiated by using friable callus in B5 liquid medium supplemented with same growth regulators in 100 ml flasks. The cultures were kept under continuous agitation at 110 rpm in an orbital shaker (Yiheng scientific instrument, Shanghai,China) and incubated at 25 ^{o}C, with a 16 h photoperiod (40 µmol m^{-2} s^{-1}).

Optimization of culture conditions

Cells were separated from the media by passing them through a 0.45 µm stainless steel sieve under sterile conditions. 2 g cells (fresh weight, FW) were cultured in 100 ml flasks containing 40 ml of B5 medium supplemented with 3 % (w/v) sucrose with pH of 5.6 ± 0.2 Culture conditions were same as above.

To study the effects of plant growth regulators on biomass accumulation and chlorogenic acid production, the medium was supplemented with various concentrations of growth regulators including 6-BA (0.5, 1.0, 2.0 mg/L), 2,4-D (0.5, 1.0, 2.0 mg/L), 1-naphthaleneacetic acid (NAA) (0.5, 1.0, 2.0 mg/L) or in combination of 6-BA (2.0 mg/L) + 2,4-D (0.5, 1.0, 2.0 mg/L), 6-BA (2.0 mg/L) + NAA (0.5, 1.0, 2.0 mg/L). A time-course test was conducted for biomass and chlorogenic acid production at 3-days intervals for 25 days. To determine optimal inoculum density, different amount of inoculant (25, 50, 75, 100 and 125 g/L) were tested for the production of biomass and chlorogenic acid. In another set of experiments, the effect of various strengths of B5 medium (1/4, 1/2, 3/4, 1, and 3/2) were tested for the production of biomass and chlorogenic acid. The effect of different sucrose concentrations (10, 20, 30, 40 and 50 g/L) was tested. The effect of different pH (5.0, 5.5, 6.0, 6.5 and 7.0) was also assessed for the biomass accumulation and chlorogenic acid production. After optimization, different concentrations (0, 10, 50, 100, 150 and 200 mg/L) of phenylalanine (metabolic precursor of chlorogenic acid) were added to the suspension culture system. Production of chlorogenic acid and accumulation of biomass were determined after 3 weeks.

Determination of cell biomass

The growth of cell cultures was measured by determining dry weight (DW). The cell cultures were filtered under vacuum and weighed to determine FW. Fresh cells were dried at 50oC in a vacuum oven overnight until they reached a constant weight, and then DW was recorded.

Extraction and HPLC analysis of chlorogenic acid

The preparation of *L. macranthoides* Hand.-Mazz. "Yulei1" extracts was done according to

the method described by Wang *et al* [14] with minor modifications. 100 mg callus powder sample was put into a 20 m test tube which contained 10 ml 60 % methanol, and was then incubated at 60 ^{o}C for 1h in an ultrasonic extractor. Each sample was extracted twice. The 60 % methanol extracts were filtered through injector and membrane filter, and then used for chlorogenic acid determination by HPLC analysis by the method described previously with minor modification [1]. The dissolved extracts were filtered through 0.22 µm membrane filter before loading into the HPLC system. Chromatography was carried out on C18 analytical column at 35 °C. The mobile phase consisted of two solvent components: glacial acetic acid water (pH = 2.6) and methanol. The flow ratio of two solvent components was 77 % glacial acetic acid water and 23 % methanol and the flow rate was 1 ml/min. The wavelength used to detect chlorogenic acid was 324 nm. 10 microliter of different concentrations (0.04 - 0.20 mg/mL) of chlorogenic acid standard was loaded into HPLC system for construction of the calibration curve by plotting the peak areas (Y) against chlorogenic acid concentrations (X). Each extract powder was dissolved in 60 % methanol to a concentration of 10 mg/ml, and 10µL of the crude extract was loaded into the HPLC system for identification and quantification of chlorogenic acid. The content of chlorogenic acid in *L. macranthoides* Hand.-Mazz. "Yulei1" was determined from the corresponding calibration curve and the content was expressed as milligram of chlorogenic acid per gram of *L. macranthoides* Hand.-Mazz. "Yulei1". The peak area of chlorogenic acid in each extract was mean of three parallel measurements for chlorogenic acid quantification. The linear regression equation was calculated as: Y = aX + b, where a = 2.063574; b = 1.104074; R^2 = 0.9998557; R = 0.9999278, which showed good linear regression within the test ranges (0.04 ~ 0.20 mg/mL). The chromatography peak was identified by comparing the retention time with that of chlorogenic acid standard.

Statistical analysis

All experiments were conducted in a completely randomized design and were repeated twice. Each treatment consisted of three replicates. Mean value of various treatments was subjected to analysis of variance (ANOVA) and significant difference was separated using Duncan's Multiple Range Test (DMRT). SPSS software was used to determine significant difference at $p \leq 0.05$. Data are presented as mean ± standard error (SE).

RESULTS

Chlorogenic acid

The HPLC chromatogram represents chlorogenic acid from 60 % methanol extracts (Figure 1a). The peaks labelled with CA were chlorogenic acid because the retention time (6.29 min) was identical with that of chlorogenic acid reference (Fig 1b).

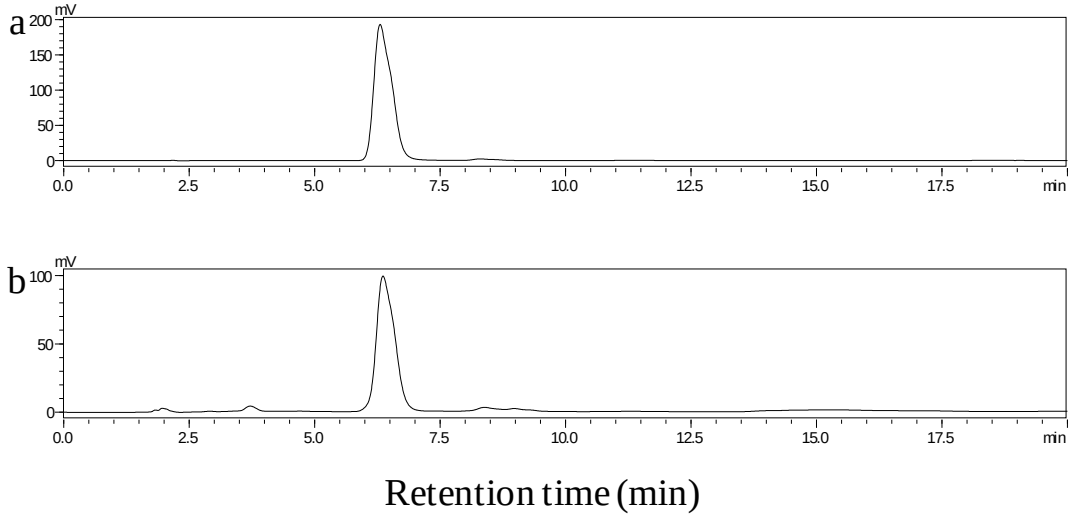

Figure 1: HPLC analysis of chlorogenic acid. ***Note:*** *'*a' = representative HPLC chromatogram of chlorogenic acid extracted from *L. macranthoides* Hand.-Mazz. "Yulei1" cell suspension culture; 'b' = representative HPLC chromatogram of chlorogenic acid standard

Effect of varying concentrations of auxin and cytokinin on biomass accumulation and chlorogenic acid production

Biomass accumulation and chlorogenic acid content were different with varied concentrations of hormones. The production of chlorogenic acid decreased with increased concentration of 2, 4-D. Increased 6-BA concentration resulted in higher production of chlorogenic acid (Fig 2a). The maximum accumulation of chlorogenic acid was 21.86 mg/g DW when medium was supplemented with 2.0 mg/L of 6-BA. The production of biomass increased with the increase of 2, 4-D or 6-BA concentration for single hormone experiment, while different concentrations of NAA caused limited changes in biomass accumulation (Figure 2b). 6-BA was the most effective hormone in increasing biomass accumulation under different concentrations. The largest accumulation of biomass was 15.26 g/L DW in medium supplemented with 2.0 mg/L6-BA (Fig. 2b). For combination of growth hormones, the highest accumulation of biomass (17.94 g/L DW) was observed with the cultures supplemented with 2.0 mg/L2,4-D + 2.0 mg/L6-BA.The maximum production of chlorogenic acid (19.44 mg/g DW) was observed with the cultures supplemented with 2.0 mg/L6-BA + 0.5 mg/L 2,4-D (Figure 3).High chlorogenic acid productivity did not always correspond to high concentration of hormones. It appears that higher concentration of 2,4-D and NAA inhibited chlorogenic acid production when combined with 2.0 mg/L6-BA. The production of biomass increased accompanying the increase concentration of 6-BA or in combination with 2,4-D and NAA.

Growth kinetics of *L. macranthoides* cell suspension cultures

The growth curve of cultured suspension cells showed S-types (Figure 4).The maximum accumulation of biomass (19.08 g/L) was reached at 15 days and topmost accumulation of chlorogenic acid (17.24 mg/g DW) was also reached at 15 days. It is clear that the biomass growth closely correlated with chlorogenic acid accumulation according to the time courses.

Effect of inoculum density on biomass accumulation and chlorogenic acid production

To determine the optimal amount of inoculum for cell culture, different densities of inoculum from 25 to 125 g/L were tested (Figure 5). Results showed that varying densities of inoculum did not have strong impact on biomass accumulation. 75 g/L was found to be suitable as this density yielded maximum biomass (17.16 g/L). However, inoculum density affected chlorogenic acid production. Chlorogenic acid was lowest when cell culture was started with 25 g/L of inoculum 50 and 75 g/L inoculum yielded higher content of chlorogenic acid than other conditions, and 50 g/L inoculum had the highest production of chlorogenic acid (16.02 mg/g DW).

Effect of different concentration of B_5 media on biomass accumulation and chlorogenic acid production

In this study, we tested the effect of 1/4, 2/4, 3/4, 4/4 and 6/4 strengths of B_5 media on biomass accumulation and chlorogenic acid production. The results showed that the 6/4 B_5 media medium favored the accumulation of biomass (13.76 g/L),

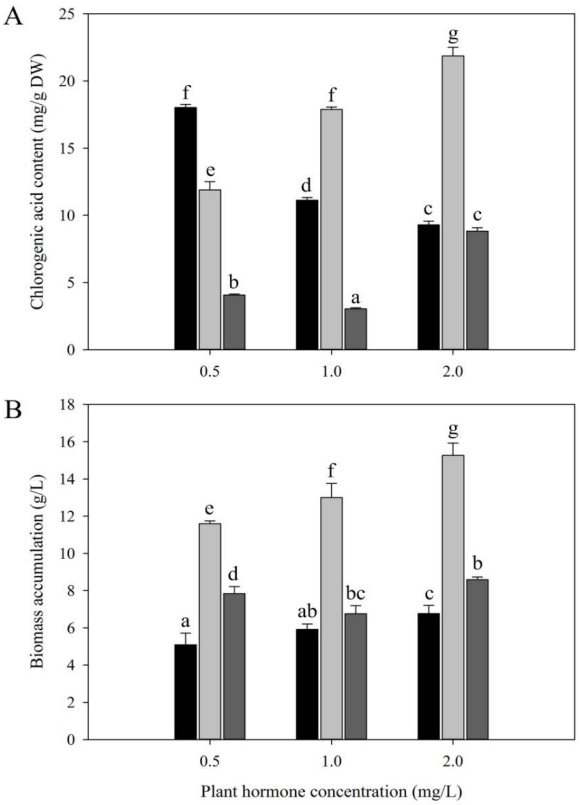

Figure 2: Effect of plant hormone concentrations on chlorogenic acid content (A) and biomass accumulation (B) in cell suspension cultures; Cells (2 g) were cultured in 40 ml of B_5 medium supplemented with different concentration of 6-BA, 2,4-D or NAA for 3 weeks. Data represent mean values ± SE of three replicates; each experiment was repeated twice. Mean values with different letters are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT). (2,4-D, 6-BA, NAA)

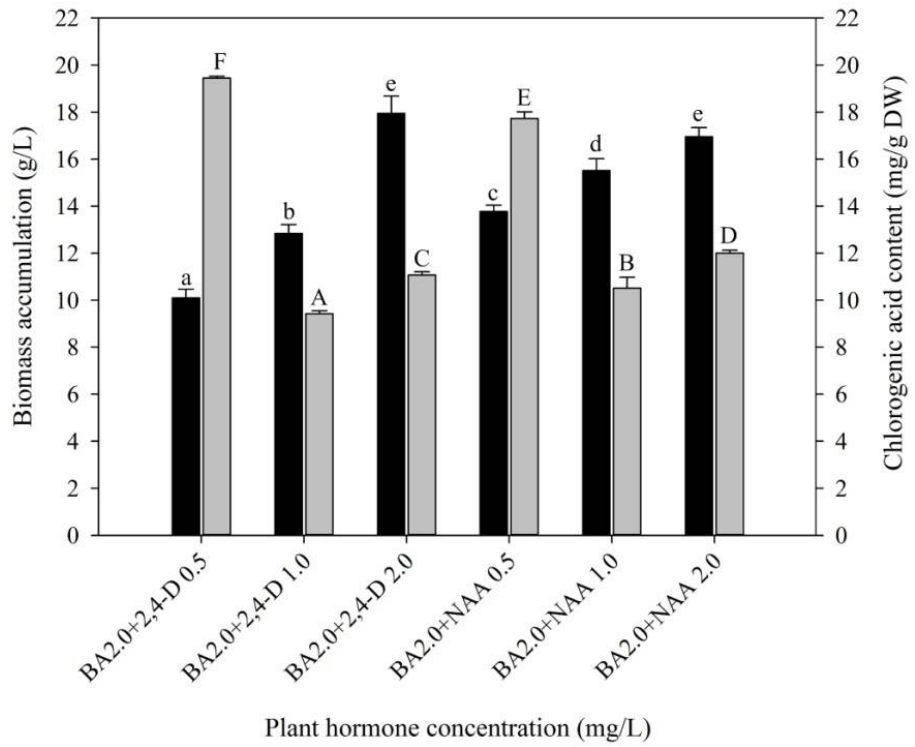

Figure 3: Effect of combination of plant hormones on chlorogenic acid content and biomass accumulation in cell suspension cultures. Cells (2g) were cultured in 40 ml of B_5 medium supplemented with various combinations of hormones for 3 weeks. Data represents mean values ± SE of three replicates. Each experiment was repeated twice. Mean values with different letters are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT). (■ DW, dry weight; ■ CA, chlorogenic acid)

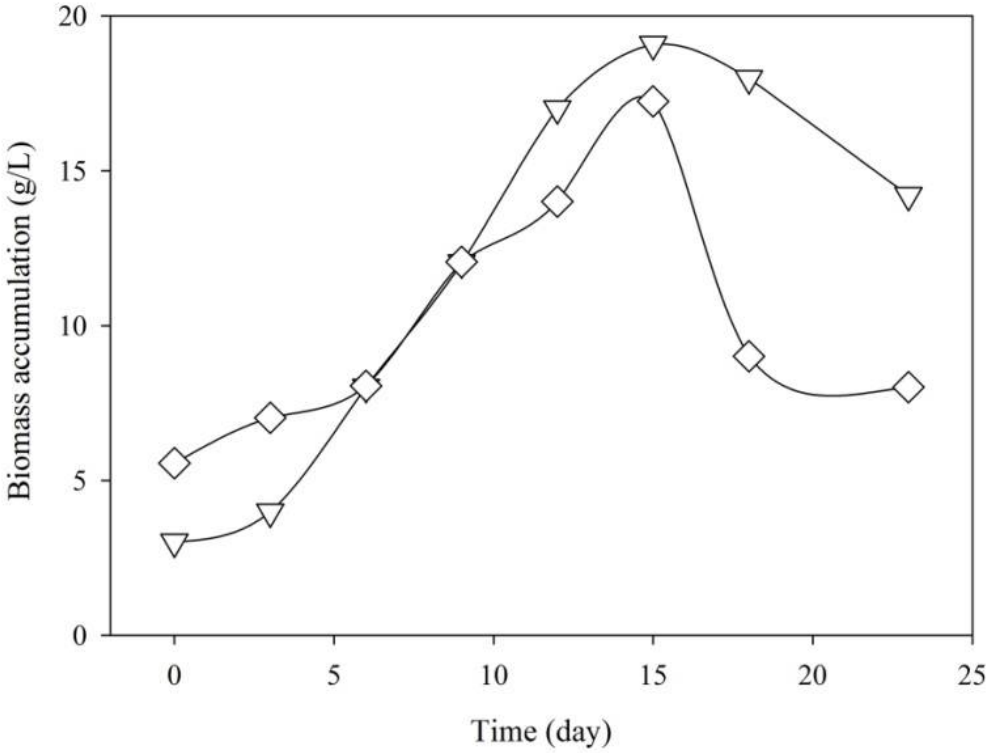

Figure 4: Biomass accumulation and chlorogenic acid production curve of suspended cultured cells. v = DW (dry weight); ◊ = CA (chlorogenic acid)

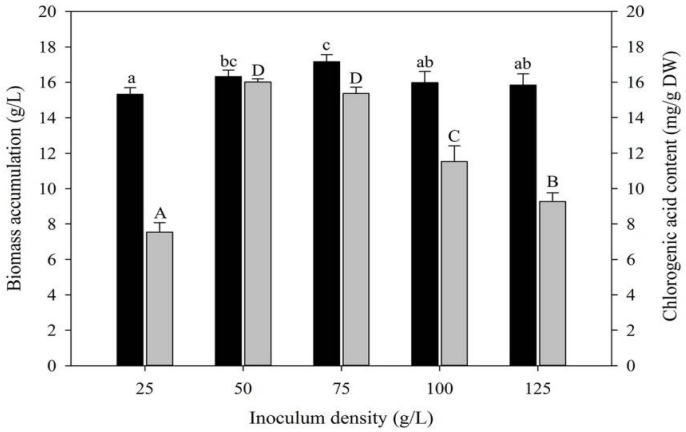

Figure 5: Effect of inoculum density on biomass accumulation and chlorogenic acid production in cell suspension cultures. Cells (2 g) were cultured in 40 mL of B_5 medium supplemented with 2.0 mg/L 2.4-D and 0.5 mg/L 6-BA for 3 weeks. Each experiment was repeated twice. Data are the mean ± SE of three replicates. Mean values with different letters are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT). (■ DW, dry weight; ■ CA, chlorogenic acid)

and the production of biomass increased with increased strength of B_5 (Fig. 6). No significant changes were observed except for 3/4 and 4/4 strength. In term of chlorogenic acid, the production of chlorogenic acid increased with the increase of B_5 from 1/4 to 4/4 strength. The highest production of chlorogenic acid (12.06 mg/g DW) was observed in 4/4 strength B_5 media (Figure 6), and then it decreased when the concentration was beyond 4/4. For optimal chlorogenic acid production and biomass accumulation, 4/4 strength B5 is best choice.

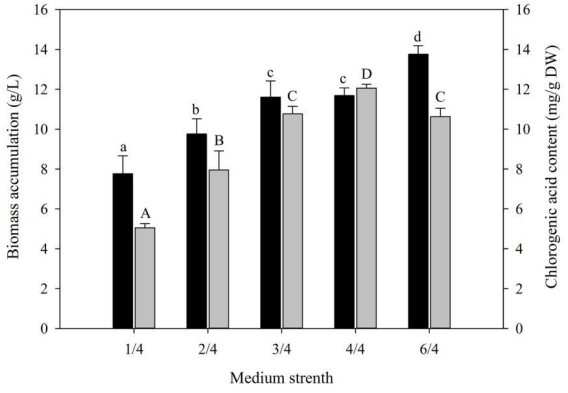

Figure 6: Effect of different medium strength on biomass accumulation and chlorogenic acid production in cell suspension cultures. Cells (2g) were cultured in 40 ml of B_5 medium supplemented with 2.0mg/L 2.4-D and 0.5 mg/L6-BA for 3 weeks. Each experiment was repeated twice. Data are the mean±SE of three independent measurements. Mean values with different letters are significantly different at P≤0.05 according to Duncan's multiple range test (DMRT). (■ DW, dry weight; ■ CA, chlorogenic acid)

Effect of different concentrations of sucrose on biomass accumulation and chlorogenic acid production

The biomass production increased with an increase in sucrose concentration however, (Figure 7) no significant changes were observed for the production of biomass between 10 and 20 g/L, also 40 and 50 g/L of sucrose. 50 g/L sucrose was found to result in highest biomass accumulation of 16.60 g/L. However, accumulation of chlorogenic acid reached highest of 14.22 mg/g DW with cultures supplemented with 30 g/L sucrose as 40 and 50 g/L sucrose inhibited chlorogenic acid production.

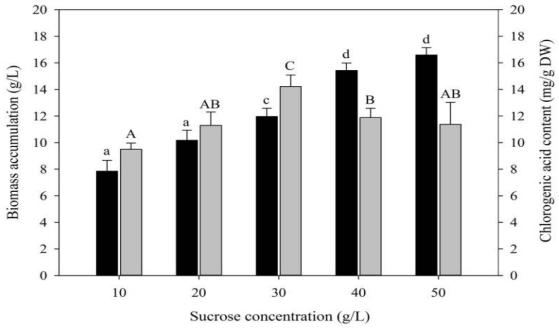

Figure 7: Effect of different sucrose concentrations on biomass accumulation and chlorogenic acid production in cell suspension cultures. Cells (2 g) were cultured in 40 ml of B_5 medium supplemented with 2.0 mg/L 2.4-Dand 0.5 mg/L 6-BA for 3 weeks. Each experiment was repeated twice. Data are the mean ± SE of three independent measurements. Mean values with different letters are significantly different at P≤0.05 according to Duncan's multiple range test (DMRT). (■ DW, dry weight; ■ CA, chlorogenic acid)

Effect of pH on biomass accumulation and chlorogenic acid production

To test the effect of pH, B_5 medium was adjusted to different pH from 5 to 7. pH had moderate effect on biomass accumulation and highest accumulation was observed when the medium pH was at 5.5 (6.52 g/L) and 6.0 (6.76 g/L). However, maximum production of chlorogenic acid content (19.11 mg/g DW) was recorded when the initial medium pH was 5.5. High and low medium pH did not favor the biomass accumulation and chlorogenic acid production (Figure 8). Chlorogenic acid still accumulated in cultured cells when pH was 7.0.

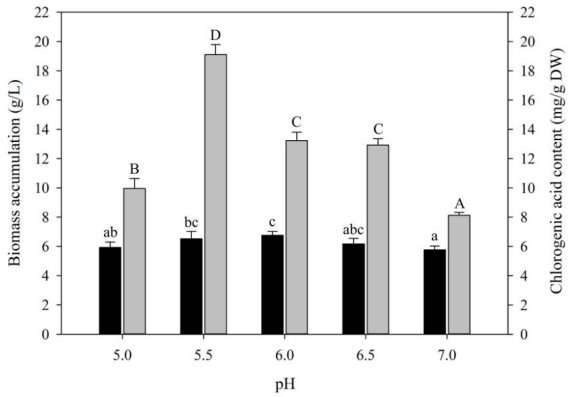

Figure 8: Effect of different hydrogen ion concentration (pH) on biomass accumulation and chlorogenic acid production in cell suspension cultures. Cells (2g) were cultured in 40 ml of B_5 medium supplemented with 2.0mg/L 2.4-D and 0.5 mg/L 6-BA for 3 weeks. Each experiment was repeated twice. Data are the mean ± SE of three independent measurements. Mean values with different letters are significantly different at P≤0.05 according to Duncan's multiple range test (DMRT). (■ DW, dry weight; ■ CA, chlorogenic acid)

Effect of phenylalanine on biomass accumulation and chlorogenic acid production

Phenylalanine was applied at concentration 10 mg/L – 50 mg/L and no significance difference in biomass was observed when compared with the control. (Fig 9). When the concentration exceeded 50 mg/L, the biomass gradually decreased with the increasing concentration of phenylalanine. (Figure 9). The experiment indicated that phenylalanine inhibited cell growth at high concentration. However, chlorogenic acid content was increased significantly with the increased concentration of phenylalanine above 50 mg/L. The maximum production of chlorogenic acid (18.00 mg/g DW) was observed when concentration of phenylalanine was 200 mg/L. Conversely, chlorogenic acid content remained the same as control when phenylalanine concentration was less than 50 mg/L (Figure 9).

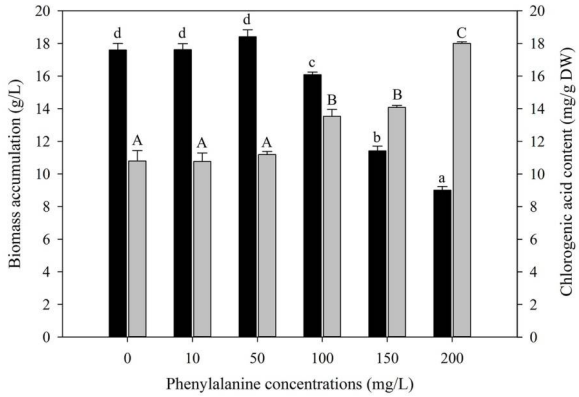

Figure 9: Effect of different phenylalanine concentrations on biomass accumulation and chlorogenic acid production in optimized cell suspension cultures. Cells (2g) were cultured in 40 ml of B_5 medium supplemented with 0.5mg/L 2.4-D and 2.0 mg/L 6-BA, pH of 5.5 for 3 weeks. Each experiment was repeated twice. Data are the mean ± SE of three independent measurements. Mean values with different letters are significantly different at $p \leq 0.05$ according to Duncan's multiple range test (DMRT). (■ DW, dry weight; ■ CA, chlorogenic acid)

DISCUSSION

As *Lonicera macranthoides* Hand.-Mazz. "Yulei1" is a new variety of honeysuckle and establishment of cell suspension cultures of "Yulei1" has not been reported. This study attempted to investigate the effect of biomass accumulation and chlorogenic acid production in cell suspension cultures using various combinations of growth hormones, inoculum density, B_5 medium strength, sucrose concentrations, hydrogen ion concentration (pH) and phenylalanine concentrations. The results of the current study pave the way for the production of chlorogenic acid on a massive scale.

The concentration of auxin or the auxin/cytokinin ratio dramatically alters both the growth and the product formation in cultured plant cells [15]. Thus, the effect of plant growth regulators on cell growth and chlorogenic acid production were tested. High accumulation of biomass and chlorogenic acid content were observed when 6-BA concentration was 2.0 mg/L. For various combinations of growth hormones, 2.0 mg/L 6-BA combined with 0.5 mg/L NAA would lead optimized cell growth and chlorogenic acid production. Wang et al [14] observed a similar

trend in *E. ulmoides* in suspension culture. Their results showed that 6-BA was the ideal hormone for chlorogenic acid accumulation.

Furthermore, maximum accumulation of biomass and chlorogenic acid were observed after 15 days of suspension cultures. Similar trend was observed by Wang et al [14] using suspension culture of *E. ulmoides*. However in the later study, the maximum accumulation of biomass was reached at 21 days. This difference may have arisen due to different plant material and culture conditions used. Results of the current study for inoculums density showed that 50 and 75 g/L of inoculum was ideal for both biomass accumulation and chlorogenic acid production, which is similar to the conclusion that moderate high inoculum size improves secondary metabolites as described by Nagella & Murthy [16].

Previous studies suggested that composition of medium nutrients is important to achieve optimal accumulation of metabolites in cultured cells [10]. In this study, for optimal chlorogenic acid production and biomass accumulation, 4/4 strength, B_5 is the best choice.

For sucrose concentrations, these results were comparable with that described by Wang *et al*. The production of biomass increased with the increase of sources concentration, and 30 g/L sucrose is the ideal carbohydrate source for chlorogenic acid accumulation.

In terms of pH, the maximum production of chlorogenic acid content was recorded when the initial medium pH was 5.5. Chlorogenic acid still accumulated in cultured cells when pH was 7.0. In other plant species, such as *E. ulmoides*, highest accumulation of chlorogenic acid content was observed when the medium pH was 5.3, which is similar to the current results. However, Wang et al documented that chlorogenic acid production was turned off when the medium pH was adjusted to 5.8 [14].

Phenylalanine, a precursor of chlorogenic acid metabolism, also has influence on biomass accumulation and chlorogenic acid production. The research result showed that chlorogenic acid contents were significantly increased with increase in concentration of phenylalanine. Wang *et al* reported that the total alkaloid of the *Fritillaria cirrhosa* D. Don cultures was significantly improved by adding different concentration of phenylalanine [17]. Similarly, Liang [18] found that the contents of daidzein in cell suspension cultures of soybean were creased when 10 µmol/L phenylalanine were added in the culture system for 48 h. These are consistent with our results. Perhaps, phenylalanine can be used as a precursor to promote plant secondary metabolite accumulation.

CONCLUSION

The findings of this study that cell suspension cultures of *L. macranthoides* Hand.-Mazz. "Yulei1" have been successfully established as suitable for the production of chlorogenic acid. These findings constitute a basis for the production of chlorogenic acid on a massive scale using bioreactor cultures of *L. macranthoides*. This will particular be useful to the pharmaceutical industries to demands for the compound, especially in China.

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CONFLICT OF INTEREST

No conflict of interest associated with this work.

CONTRIBUTION OF AUTHORS

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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