African Journal of Biotechnology Vol. 10(40), pp. 7831-7839, 1 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.638
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Effects of several physiochemical factors on cell growth and gallic acid accumulation of *Acer ginnala* Maxim cell suspension culture

Jie Dong and Yaguang Zhan*

College of Life Sciences, Northeast Forestry University, Harbin 150040, Heilongjiang, P.R. China.

Accepted 2 June, 2011

The production of gallic acid in cell suspension culture of *Acer ginnala* Maxim was studied. Some physiochemical factors and chemical substances effect on the cell growth and the production of gallic acid were investigated. Cells harvested from plant tissue culture were extracted and applied to high performance liquid chromatography to measure gallic acid content. 0.008 mg.L^{-1} TDZ and 0.1 mg.L^{-1} BA was optimal for the cell growth. 0.004 mg.L^{-1} TDZ and 0.1 mg.L^{-1} BA was best for the production of gallic acid. Maintaining the initial pH value at 5.8 was most suitable for gallic acid accumulation in *A. ginnala* Maxim cell suspension cultures. To satisfy the condition of mass-producing gallic acid in the suspension culture, the adapted inoculum quantity was 30 g.L^{-1}. The results also provided evidence that the optional culture period was 7 days with light.

Key words: *Acer ginnala* Maxim, gallic acid, cell growth, suspension culture, physiochemical factor.

INTRODUCTION

Acer ginnala Maxim (Amur Maple) is a species of maple native to northeastern Asia from easternmost Mongolia east to Korea and Japan, and north to southeastern Siberia in the Amur River valley. It is a deciduous spreading shrub or small tree growing to 3 to 10 m tall, with a short trunk up to 20 to 40 cm diameter and slender branches. The leaves turn brilliant orange to red in autumn, and are on slender, often pink-tinged, petioles 3 to 5 cm long (Rushforth, 1999). Medicinally, *A. ginnala* has a variety of uses. The young leaves were used as a tea substitute (Kunkel, 1984). High yields of polyphenol were obtained from *A. ginnala* Maxim (Carr, 1985).

Plant secondary metabolites have found applications in pharmaceutical industries, cosmetics, biopesticides and agrochemicals, flavours or food additives, odours and fragrances, and natural pigments (Dicosmo and Misawa, 1995; Oksman-Caldentey and Hiltunen, 1996; Ramachandra and Ravishankar, 2002).

Gallic acid (3,4,5-trihydroxybenzoic acid) (GA) is a low molecular weight phenolic (Natasha et al., 2003). It is a naturally occurring polyphenol present in many fruits, vegetables and derivative products (José and Cristina, 2010). It has been found to be pharmacologically active as an antioxidant, antimutagenic, lipid-lowering, anti-atherosclerotic, anti-liver injury, anti-tumor, and anti-carcinogenic agent (Lin et al., 2004; Sun et al., 2010; Lv et al., 2010; Jiang and Yang, 2010; Lu et al., 2010). It is also used in the leather industry, in manufacturing gallic acid esters, example, propyl gallate which is used as an antioxidant, in the manufacture of pyrogallol (Zhang et al., 2010, Wang et al., 2009). Pyrogallol is used in staining fur, leather and hair, and also as a photographic developer (Kar et al., 1999). Gallic acid and its catechin derivatives are also present as one of the main phenolic components of both black and green tea. Esters of gallic acid have a diverse range of industrial uses, as antioxidants in food, in cosmetics and in the pharma- ceutical industry. In addition, gallic acid is employed as a source material for inks, paints and colour developers. Studies utilizing these compounds have found them to possess many potential

*Corresponding author. E-mail: yaguangzhan@126.com.cn. Tel: +86 451 82191752.

Abbreviations: **BA,** N6-Benzyladenine; 2,4-D, 2,4-dichlorophenoxy acetic acid; **DW,** dry weight; **FW**, fresh weight; **HPLC**, high performance liquid chromatography; **NAA**, naphthalenecetic acid; **WPM,** woody plant medium; **TDZ,** thidiazuron.

therapeutic properties including anti-cancer and anti-microbial properties (Ow and Stupans, 2003).

Because gallic acid was mainly separated from *A. ginnala* Maxim leaves, its production was influenced by *A. ginnala* Maxim growth and the environmental factors. Plant cell cultures are a potential source for a huge variety of useful chemical compounds. It is now widely being used as a model system to investigate the production of specific secondary products as they offer experimental advantages (Swapna et al., 2000). Cell suspension culture is widely used, especially in research as one of the plant tissue culture techniques. It has the advantages of giving homogeneity and a higher efficiency of propagation of cultured cells compared with callus culture on solidified media. In addition, from an engineering perspective, cell suspension culture has more immediate potential for industrial application than other plant tissue cultures, due to the extensive expertise that has been amassed for the treatment of submerged microbial cultures. Accordingly, cell suspension cultures for commercial use have been studied recently (Ibaraki and Kurata, 2001). Presently there has been increasing interest in exploiting *A. ginnala* Maxim cell cultures to produce the anti-cancer drug, gallic acid. The objectives of this study were to establish cell suspension cultures of *A. ginnala* Maxim for the production of gallic acid by manipulating different combination of phytohormones. The effect of the beginning pH of the medium, the subculture time and inoculation density on cell growth and gallic acid production was also studied.

MATERIALS AND METHODS

Growth measurement

Cell fresh weight (FW) was measured and then cell dry weight (DW) was measured by drying the cell cake at 60°C in an oven to a constant weight. Increasing index = W_1/W_2 (W_1: the weight of the harvest, W_2: the weight of the beginning). Results were expressed as increasing index.

Cell cultures

Seeds of *A. ginnala* Maxim were surface sterilized with 70% (v/v) ethanol for 40 s and 3% (w/v) sodium hypochloride for 15 min, and rinsed three times in sterile distilled water. Surface-sterilized seeds were germinated in 50 ml Erlenmeyer flasks containing 20 ml WPM medium supplemented with 0.5 mg.L^{-1} BA, 20% (w/v) sucrose, solidified with 0.6% agar. The pH of the medium was adjusted to 5.8 before autoclaving and cultured at 25°C in the light for two weeks. Explants were excised from cotyledon. Explants were placed in 50 ml Erlenmeyer flasks containing 20 ml WPM medium supplemented with 0.004 mg/L TDZ, 0.1 mg/L BA, 20% (w/v) sucrose, solidified with 0.6% agar (Dong et al., 2008). The pH of the medium was adjusted to 5.8 before autoclaving, and cultured at 25°C in the light for three weeks. Subculture of the induced callus was performed at the same culture condition. After 4 to 5 subcultures homogeneous, incompact green callus were obtained.

The callus were transferred to 100 ml Erlenmeyer flasks containing 50 ml of WPM liquid medium supplemented with different kinds of phytohormones, 20% (w/v) sucrose, and the pH of the medium was adjusted to 5.8 before autoclaving. The suspension culture was grown under white light on a rotary shaker at 120 rpm and was subcultured every seven days.

When the proper phytohormones were selected, some different physiochemical factors were supplied. The pH of the medium was respectively adjusted to 5.3, 5.8, 6.3, 6.8 and 7.3 before autoclaving. The suspension culture was subcultured every 7, 14, 21 and 28 days. 1, 1.5 and 2.0 g FW of callus were inoculated into each flask. Suspension cultures were incubated for 28 days, then FW, DW and content of gallic acid were measured. The cultures were grown under 12 h.day^{-1} illumination.

Chemical analysis

pH in the culture medium was determined using the pHS-3C precision pH instrument (Shanghai Precision and Scientific Instrument CO., LTD).

Extraction and HPLC analysis of gallic acid

Gallic acid was extracted from 0.1 g of dried, powdered suspension callus tissue dissolved in 2 ml methanol and 2 ml 10% (v/v) sulfuric acid. The sample was treated by ultrasound wave for 2 min and then was kept in a constant temperature water bath at 60°C for an hour, which was added methyl alcohol to 5 ml. After centrifugating for 5 min, supernatant was filtered with 0.45 μm microporous filter for further use. Gallic acid was analyzed using a reverse phase column (HiQ sil C18 W 4.6 mmΦ×250 mm); mobile phase: methanol- water (40:60); flow rate: 0.5 ml.min^{-1}; volume of injected sample: 10 μl; UV detection was carried out at 270 nm. Retention time of gallic acid was 5 min. The compound was identified by comparison with an authentic sample. The gallic acid content was expressed as the percentage of DW.

RESULTS

Effect of phytohormones on cell growth and gallic acid production in cell suspension culture

Effect of WPM medium supplemented with KT 0.25 mg.L^{-1} and different concentration of 2, 4-D is shown in Figure 1. Cell growth was generally higher at 2 mg.L^{-1} 2, 4-D and increasing index of DW was 3.25. Gallic acid production was generally higher at 2 mg.L^{-1} 2, 4-D. But 2, 4-D and KT produced a fast change in the biomass color, from light yellow to light brown and lots of suspension cells were also died. So, different combination of 2, 4-D and KT was not proper for cell growth and gallic acid production in cell suspension culture.

Effect of WPM medium supplemented with 0.2 mg.L^{-1} BA and different concentration of NAA is shown in Figure 2. The biomass production and production of gallic acid increased when NAA increased from 0 to 2 mg.L^{-1}. However, the biomass production and production of gallic acid reduced at 2.5 mg.L^{-1} NAA. So, 2.0 mg.L^{-1} NAA and 0.2 mg.L^{-1} BA was more proper when compared.

Effect of WPM medium supplemented with 0.1 mg .L^{-1} BA and different concentration of TDZ is shown in Figure3. The results provided evidence that the application of

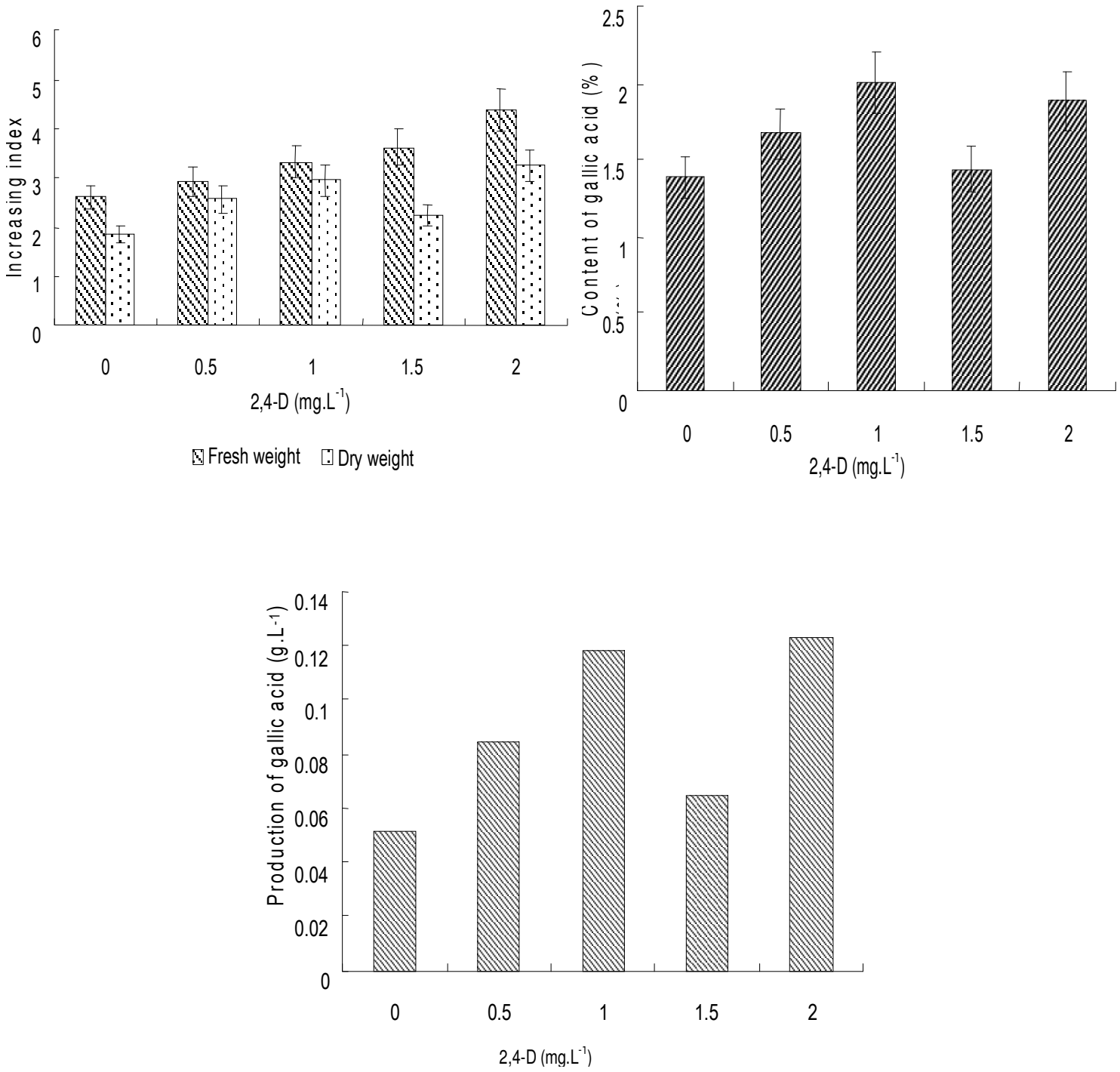

Figure 1. Effect of different combination of 2, 4-D and KT on cell growth and gallic acid production in cell suspension culture.

0.008 mg.L^{-1} TDZ and 0.1 mg.L^{-1} BA was accompanied by a substantial increase in cell growth. Increasing index of DW was 12.8. On the other hand, the production of gallic acid decreased when TDZ increased from 0 to 0.016 mg.L^{-1}. Comparing the biomass production and production of gallic acid, the best combination was 0.004 mg.L^{-1} TDZ and 0.1 mg.L^{-1} BA.

Effect of the intial pH of the medium on cell growth and gallic acid production in cell suspension culture

The initial of pH of the medium was respectively adjusted to 5.3, 5.8, 6.3, 6.8 and 7.3. As indicated in Figure 4, we observed intensive growth of the culture and high production of gallic acid when the initial of pH of the

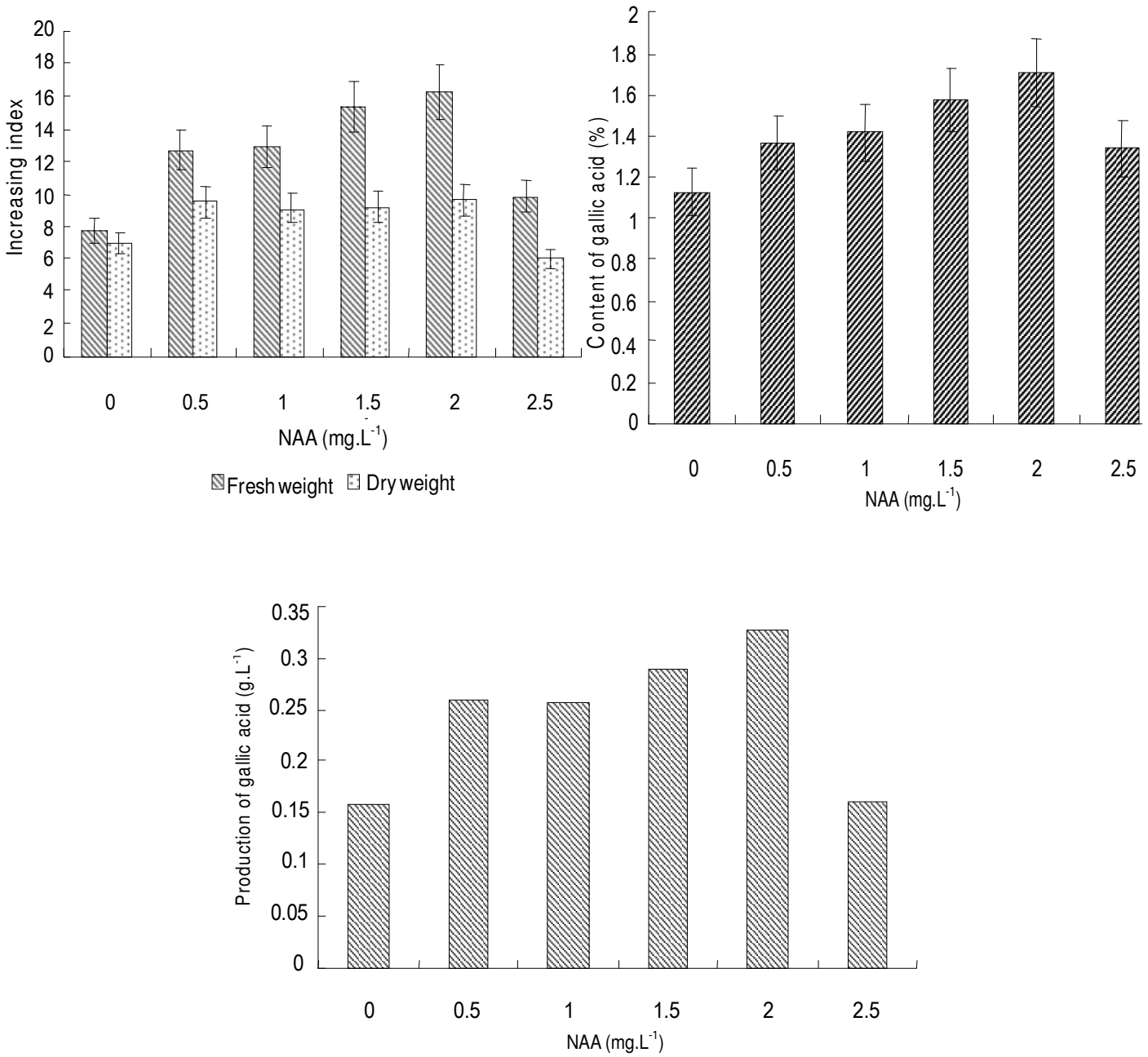

Figure 2. Effect of different combination of NAA and BA on cell growth and gallic acid production in cell suspension culture.

medium was adjusted to 5.8.

Effect of the subculture time on cell growth and gallic acid production in cell suspension culture

Subculture time plays an importance role in cell growth and gallic acid production in cell suspension culture. As indicated in Figure 5, we found that when the suspension culture was subcultured every 7 days, increasing index of FW and DW was highest (12.05 and 7.4). When the suspension culture was subcultured every 14 days the production of gallic acid was higher (0.577%). Considering both the growth and the production of secondary metabolites, we subcultured the suspension culture every 7 days.

Effect of the inoculum density on cell growth and gallic acid production in cell suspension culture

As shown in Figure 6, when the inoculum density was 30

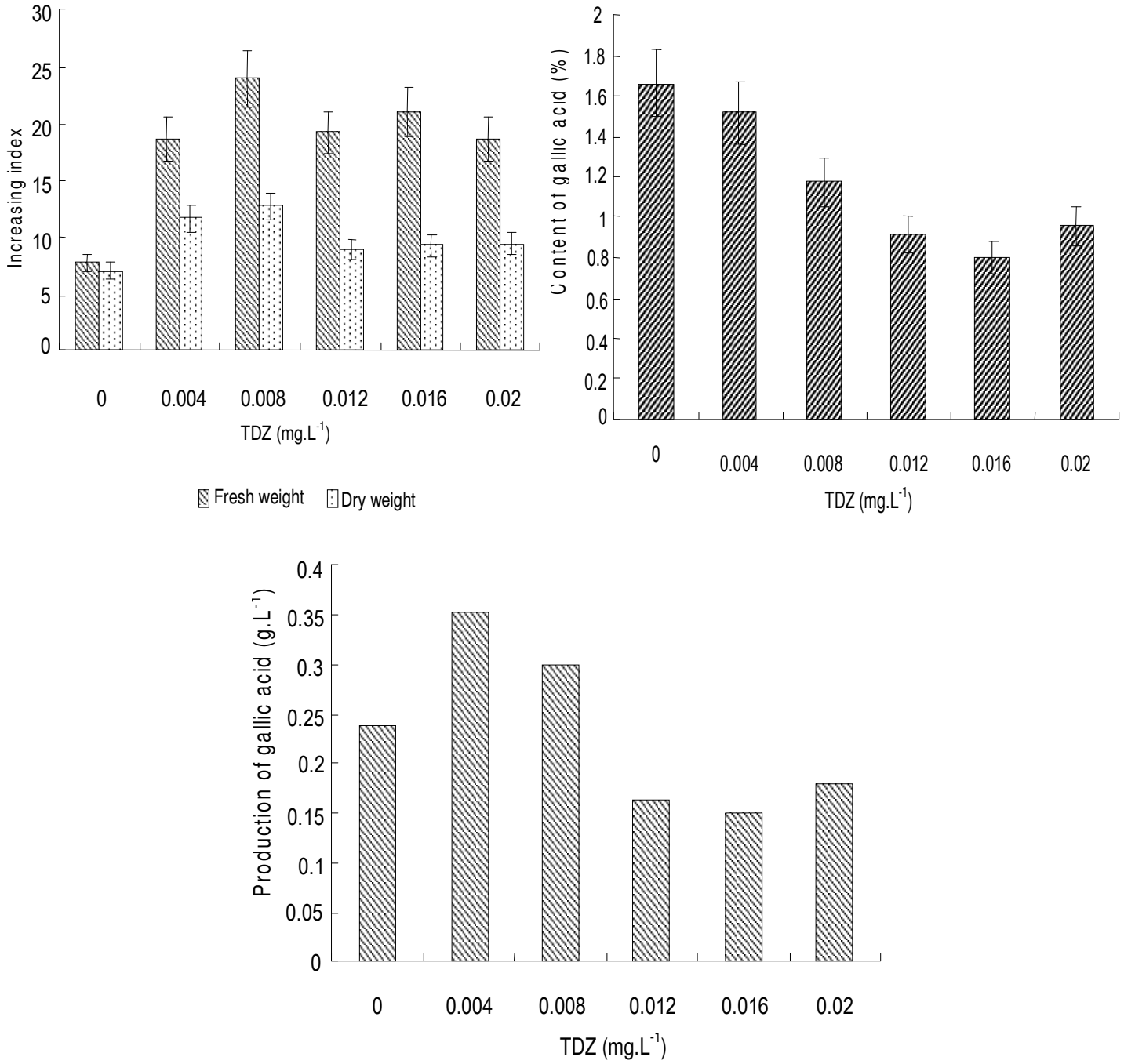

Figure 3. Effect of different combination of TDZ and BA on cell growth and gallic acid production in cell suspension culture.

$g.L^{-1}$, increasing index of FW and DW and the production of gallic acid was highest.

DISCUSSION

The cell grows and gallic acid production of *A. ginnala* Maxim is influenced by different combination of phytohormones, the initial pH of the medium, the subculture time and inoculation density. Hormones are the main materials which regulate the plant growth and the secondary metabolites formation of plant. The exogenous hormones' categories, concentration and proportion could influence on cell biomass and metabolites content in plant cell suspension culture (Dai and Li, 2007). For example, effect of 2, 4-D, IAA, IBA and NAA was examined on cell growth and berberine production in cell suspension culture of *Coscinium fenestratum* (Gaertn.) Colebr- a

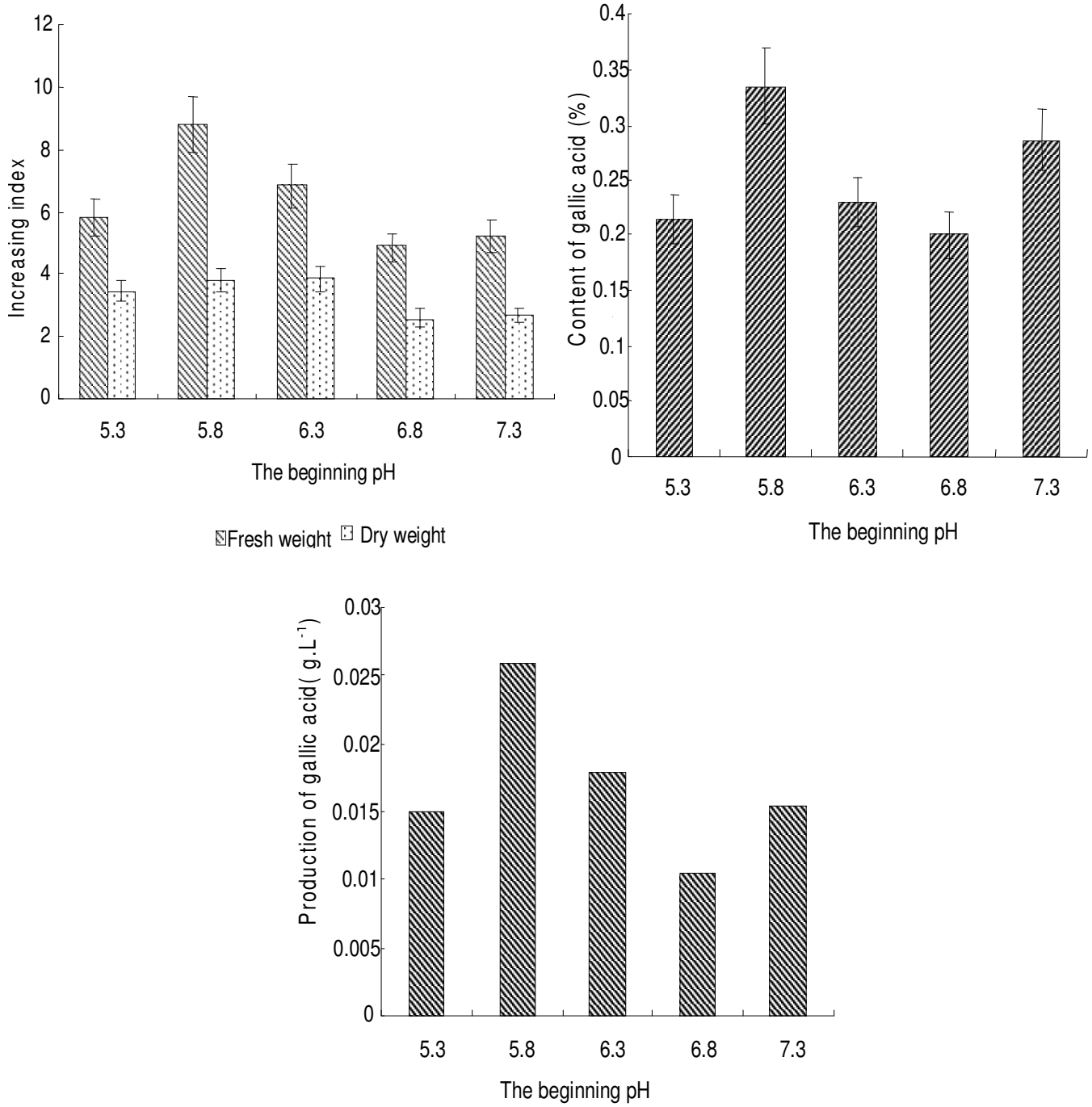

Figure 4. Effect of the beginning pH of the medium on cell growth and gallic acid production in cell suspension culture.

critically endangered medicinal liana of Western Ghat, which found that highest yield of berberine (5.79 mg/30 ml; 4.14 times to that of control) was obtained with 4 mg.L^{-1} of NAA, while the best cell growth (214.43 mg dry weight, 1.96 times to that of control) was observed in the presence of 2 mg.L^{-1} of2,4-D. IAA and IBA were not favourable for cell growth and berberine synthesis (Narasimhan and Nair, 2004). Sycamore (*Acer pseudoplatanus* L.) cell suspension cultures (strain OS) require 2, 4-D in their culture medium for normal growth (Moloney et al., 1983). The influence of phytohormones affecting jaceosidin formation was examined in callus and cell suspension cultures of *Saussurea medusa*. The best cell growth (19.70 g dry weight. L^{-1}) and jaceosidin production (0.3% dry weight) were found in combination of NAA 2 mg.L^{-1} with BA 0.2 mg.L^{-1}. For the jaceosidin

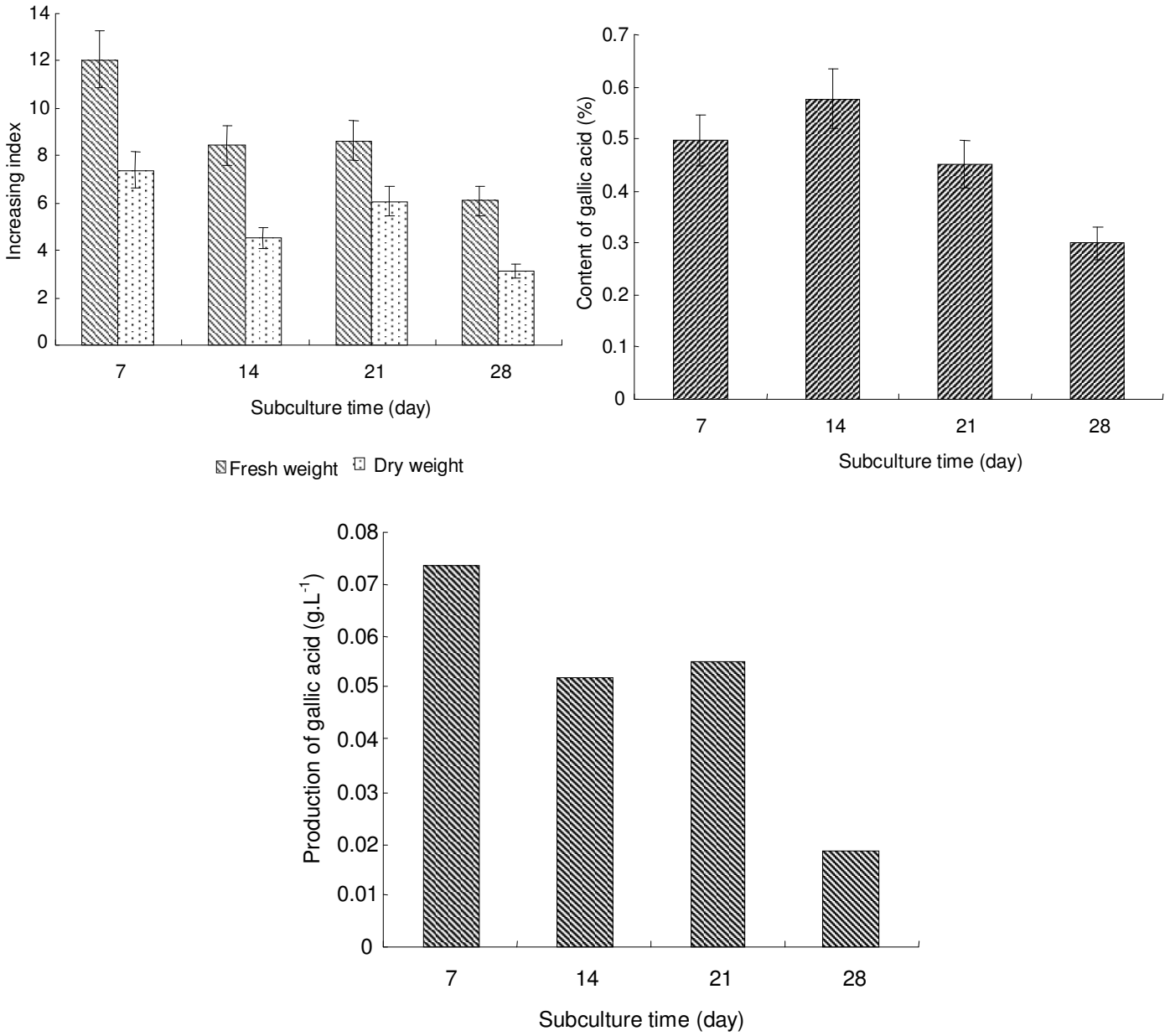

Figure 5. Effect of the subculture time on cell growth and gallic acid production in cell suspension culture.

formation, NAA was more effective of the auxins tested and 2,4-D tended to restrain the jaceosidin formation (Zhao et al., 2001). However, different species of plant cell suspension culture demand different types and concentration of hormones. In this study, highest yield of gallic acid was obtained with 0.008 mg.L^{-1} TDZ and 0.1 mg.L^{-1} BA, while the best cell growth was observed in the presence of 0.004 mg.L^{-1} TDZ and 0.1 mg.L^{-1}. 2, 4-D and KT were not favourable for cell growth and gallic acid synthesis. Similar results have been reported for callus cultures of *A. ginnala* Maxim (Li et al., 2008).

What's more, other factors, that is temperature, light, inoculum size, type of media, nitrogen and carbon source etc. also affected secondary metabolite formation in plant cell suspension culture. Fang et al. (2006) found that maintaining the pH value at the range over 5.4 to 5.8 was most suitable for isoflavone accumulation in *P. lobata* cell suspension cultures. Chattopadhyay et al. (2002) indicated that inoculum level, glucose, IAA, and pH had significant effects on growth and production of podophyllotoxin and determined the optimum concentrations of these parameters, which were pH 6.0, 1.25 mg.L^{-1} of IAA, 72 g.L^{-1} of glucose, and inoculum level of 8 g.L^{-1}. The effects of the cultivation media, plant growth regulators and inoculum size on the cell growth and 20-hydroxyecdysone production in suspension cultures of *Vitex*

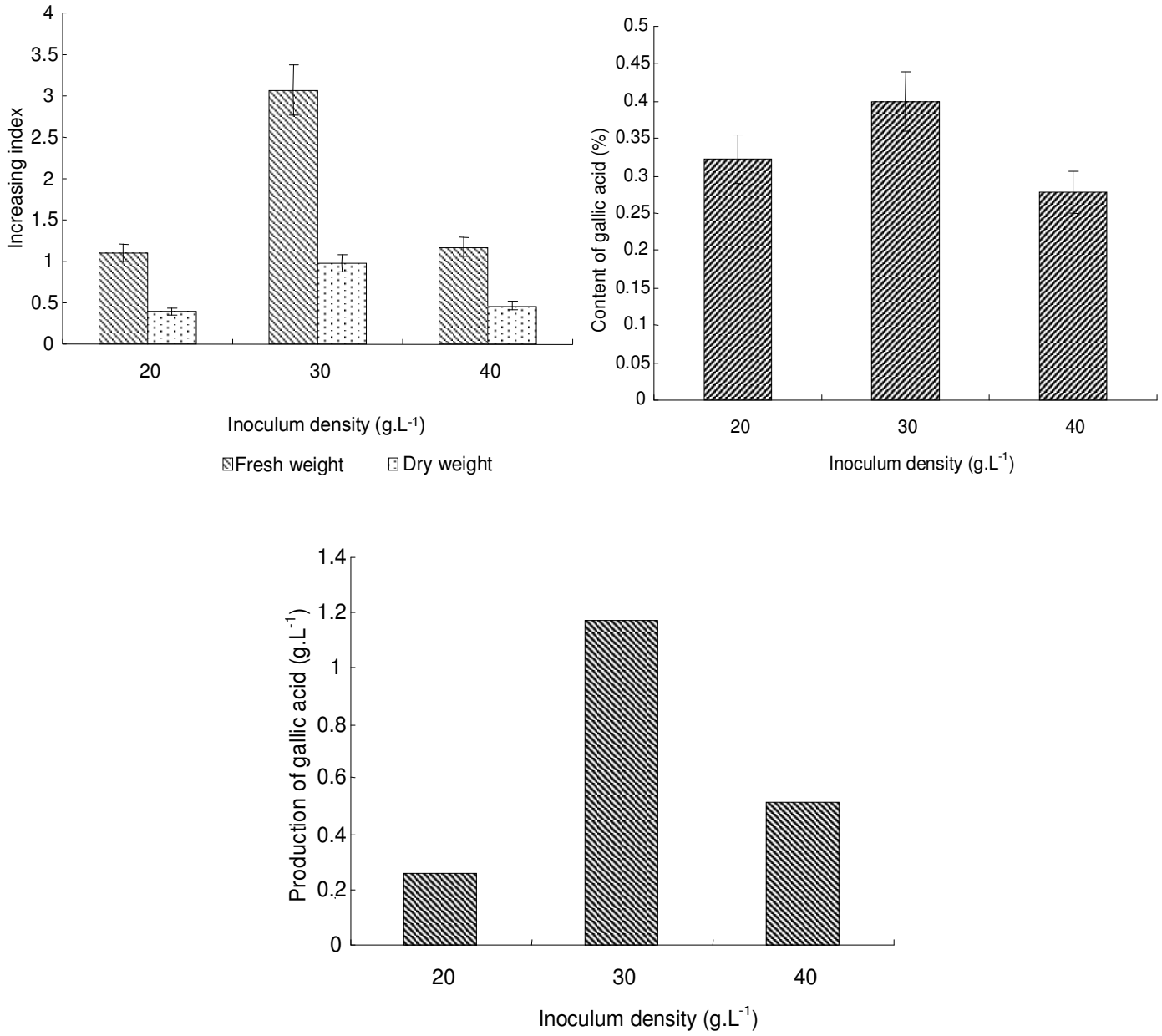

Figure 6. Effect of the inoculum density on cell growth and gallic acid production in cell suspension culture.

glabrata R. Br. were investigated. The cell growth and 20-hydroxyecdysone formation reach the highest when cells are cultured in the Gamborg's B5 medium supplemented with 2.0 mg.L^{-1} BAP (6-benzylaminopurine) and 1.0 mg.L^{-1} 2,4-D. The maximum 20-hydroxyecdysone productivity, of about 1.1 mg.L^{-1}.day^{-1} was observed in the culture with 20% packed cell volume (PCV) of inoculum size. These data also show that the increment of the inoculum size to 20% PCV could increase the productivity in 7-folds (Sinlaparaya et al., 2007). In this study, we found that maintaining the beginning pH value at 5.8 was most suitable for gallic acid accumulation in *A. ginnala* Maxim cell suspension cultures, the adapted inoculum quantity was 30 g.L^{-1} and the optional culture period was 7 days with light. Other factors will be examined in the future studies.

The instability of production of secondary metabolites is a bottleneck in the commercialization of plant cell cultures, and also, the understanding of its mechanisms or causes is still poor. This study also discovered that after repeated subculture gallic acid content existence of certain instability. Similar results have been reported in Suspension Cultures of *Vitis vinifera*. A number of mechanisms have been proposed, including inherent heterogeneity of the source explant material, genetic and epigenetic instabilities, environmental stress, lack of tissue differentiation,

and involvement of chemical signals (Jun-Ge et al., 2006).

ACKNOWLEDGEMENTS

This work was supported in part by Specialized Research Fund for the Doctoral Program of Higher Education (No.20060225010).

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