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Full Length Research Paper

High temperature effects on flavones accumulation and antioxidant system in *Scutellaria baicalensis* Georgi cells

Yuan Yuan[1#], Yunjun Liu[2#], Yujian Luo[1], Luqi Huang[1*], Shunqin Chen[1], Zhaochun Yang[1] and Shuangshuang Qin[1]

[1]Institute of Chinese Material Medical, Academy of Chinese Medical Sciences, Beijing 100700, China.
[2]Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing 100081, China.

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Scutellaria baicalensis **is a traditional Chinese medicinal plant that has long been grown in Hubei Province. However, increasing average annual temperatures in the region have made plants unsuitable for medicinal use. Two flavones, baicalin and baicalein, are the major active ingredients of *S. baicalensis*. We demonstrated that protracted heat treatment inhibited the accumulation of baicalin and baicalein as well as the activity of phenylalanine ammonla-lyase (PAL). PAL is involved in the phenylpropanoid pathway, which produces baicalin in the plant. Heat treatment also affected the activities of the antioxidant enzymes such as catalase (CAT), superoxide dimutase (SOD) and peroxidase (POD). However, superoxide radical and hydrogen peroxide content of the cells were not significantly impacted by heat treatment. Cells continued growing during the protracted heat stress. Hence, the increasing temperatures in Hubei Province will very likely make *S. baicalensis* unsuitable for medical use by decreasing the flavonoid content.**

Key words: *Scutellaria baicalensis* Georgi, heat, antioxidant system, flavonoid.

INTRODUCTION

Scutellaria baicalensis Georgi is a traditional Chinese medicinal plant, whose active compounds comprise baicalin, baicalein, wogonoside, wogonin, neobaicalein, visidulin I and oroxylin A. These compounds have anti-burning, anti-tumor and anti-HIV activity (Blach-Olszewska et al., 2008). Temperature is an important environ-mental factor that may affect the medicinal quality of *S. baicalensis* (Li, 2008). The species has long been grown in Hubei Province, where increasing temperatures have made other plants unsuitable for medicinal purposes. The Intergovernmental Panel on Climate Change Working Group II reported that many natural systems are being affected by regional climate change, particularly temperature increases (Rosenzweig, 2008), and Hubei Province is one of the impacted regions.

High temperature is a significant abiotic stress that can limit plant growth and productivity; heat stress may inhibit photosynthesis, damage cell membranes and cause senescence and cell death (Xu et al., 2006). One mechanism of injury under high temperature involves the over-production of reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydroxyl radicals (OH), hydro-gen peroxide (H_2O_2) and single oxygen ($^1O_2$) (Mittler, 2002). To control ROS in their cells, plants produce several enzymatic ROS-scavenging enzymes, including superoxide peroxidase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione peroxidase (GPX). Ascorbate, glutathione, carotenoids and tocopherols are major non-enzymatic antioxidants. Previous studies have shown that high concentrations of antioxidant enzymes and antioxidants contribute to high-temperature tolerance in plants (Yin et al., 2008).

Flavonoids are important secondary plant metabolites that also play important roles as antioxidants that combat ROS toxicity (Morimoto et al., 1998). Flavonoid accumu-lation protects plants against various stressful conditions, including cold treatment (Lillo et al., 2008) and freezing (Hannah et al., 2006). In *S. baicalensis*, baicalein is

*corresponding author. Email: huangluqi@263.net

#These authors contributed equally to this work.

Table 1. Cell samples recovered from heat treatment.

Sample	40℃		20 to 25℃	
	Light time per day (h)	Duration time (day)	Light time per day (h)	Duration time (day)
CK			24	20
H1	24	5	24	15
H2	24	10	24	10
H3	24	15	24	5
H4	16	20	8	20

involved in scavenging H_2O_2, a substrate of peroxidases (Morimoto et al., 1998). Cellular senescence is accompanied by increases in intracellular ROS concentration and the accumulation of DNA damage. Activities and capacities of antioxidant systems in plant cells decline during aging, leading to a gradual loss of the pro-oxidant/antioxidant balance and accumulated oxidative damage (Kumaran et al., 2009). Total flavonoid accumulation has been reported in senescent *S. baicalensis* (Li, 2008).

We investigated the long-term effects of heat treatment on *S. baicalensis* cells to determine how the increasing average annual temperatures in Hubei Province may be affecting the medicinal properties of *S. baicalensis*.

MATERIALS AND METHODS

Plant material and treatments

Sterile *S. baicalensis* seedlings were grown in bottles on Murashige and Skoog (MS) basal medium containing 30 g/l sucrose and 8 g/l agar. Calli were induced from shoot stem segments on solid MS medium containing 0.25 mg/l kinetin (KT) and 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). Cells in the suspension culture were obtained by incubating 4-week-old calli in liquid MS medium containing 0.5 mg/l 2,4-D and 3 mg/l thidiazuron (TDZ) at 25℃ under dark conditions. Cell suspensions were subcultured every 4 weeks in liquid MS medium under the same culture conditions. Subcultured cells were grown separately at 25 and 40℃, both with a 16 h-light photoperiod. Cells were sampled four times (20, 22, 24 and 26 days) after subculture. Cells were also grown under high temperature and subsequently were transferred to normal temperature (Table 1). The sampled cells were centrifuged at 15,000 × *g* for 20 min at 4℃, rinsed three times in distilled water and then stored at –80℃ until they were used in experiments.

Superoxide radical (O_2^-) production rate and H_2O_2 concentration

O_2^- production rate was measured following the methods of Ke et al. (2002) with slight modifications. Briefly, 0.1 g fresh cell suspension was extracted with 1.5 ml 50 mM potassium phosphate buffer (pH 7.8). The supernatant was mixed with an equal volume of a solution containing 1 mM hydroxylammonium chloride, 17 mM 4-aminobenzenesfonic acid and 7 mM a-naphthylamine. The mixture was maintained for 20 min at 25℃ and specific absorption was measured at 530 nm. H_2O_2 concentration was measured by monitoring the absorbance of a titanium peroxide complex at 415 nm, following the methods of Patterson et al. (1984).

Analysis of enzyme activities

Fresh cell suspensions were collected and homogenized in 5 ml 50 mM sodium phosphate buffer (pH 7.0 for CAT and pH 7.8 for SOD and POD) containing 1% (w/v) polyvinylpyrrolidone (PVP) and 0.1 mM Na_2EDTA. The homogenate was filtered through four layers of cheesecloth and centrifuged at 15,000 × *g* for 20 min. The supernatant was used to determine the enzyme activities and protein concentration. Extractions and enzyme assays were performed at 4 and 25℃, respectively.

SOD activity was measured spectrophotometrically as described by Beyer and Fridovich (1987), with one unit of SOD defined as the amount required to inhibit photoreduction of nitroblue tetrazolium by 50%. CAT activity was assayed following Clairborne (1985), with decomposition of H_2O_2 followed by a decline in absorbance at 240 nm for 2 min. One unit of catalase was converted to 1 mmol H_2O_2 min^{-1}. POD activity was determined following Chance and Maehly (1955), using guaiacol as an electron donor. Protein concentrations in the enzymatic extractions were measured following Bradford (1976).

PAL activity was determined as described by Ke and Saltveit. (1986). Homogenized samples were extracted with 0.1 M sodium borate (pH 8.8). The change in absorbance at 290 nm was monitored in 1 cm light path cells in 10- to 15-min intervals for 30 min at 30℃. Under these conditions, an absorbance change of 0.01 units was found to be equivalent to the production of 1 µg/ml cinnamic acid.

β-Glucuronidase (GUS) activity of cell suspensions was measured using the 4-methylumbelliferyl-beta-D-glucoronide trihydrate (MUG) assay (Wang et al., 2002). Production of 4-methylum-belliferone (4-MU) was measured with a fluorometer (CytoFluor, Applied Biosystems, Foster City, CA, USA), determined from a standard curve.

HPLC analysis of flavonoid

A total of 100 mg powdered material was extracted for 1 h in 1 mL ethyl alcohol. The solution was passed through a membrane filter (0.2 µm) and the concentrations were determined with a high performance liquid chromatography (HPLC) system using a 1.0 mL/min flow rate. HPLC was performed in a diamonsil C_{18} column (4.6 mm × 250 mm, 5 µm). The detection wavelength was set at 280 nm and column temperature was maintained at 30℃. The mobile phase consisted of mixtures of acetonitrile–deionized water–methanoic acid in two solutions: A (21:78:1, v/v) and B (80:20:1, v/v). The initial condition was A–B (100:0, v/v) for 15 min, linearly changed to A–B (87:13, v/v) at 25 min, to A–B (52:48, v/v) at 40 min and to A–B (0:100, v/v) at 60 min. HPLC grade acetonitrile (E. Merck, Darmstadt, Germany) was used for the HPLC analysis. Peaks were identified by the retention times of standards supplied by the National Institute for The Control of Pharmaceutical and Biological Products (China). The standard solutions contained 0.208 mg/ml baicalin and 0.602 mg/ml baicalein. The injection

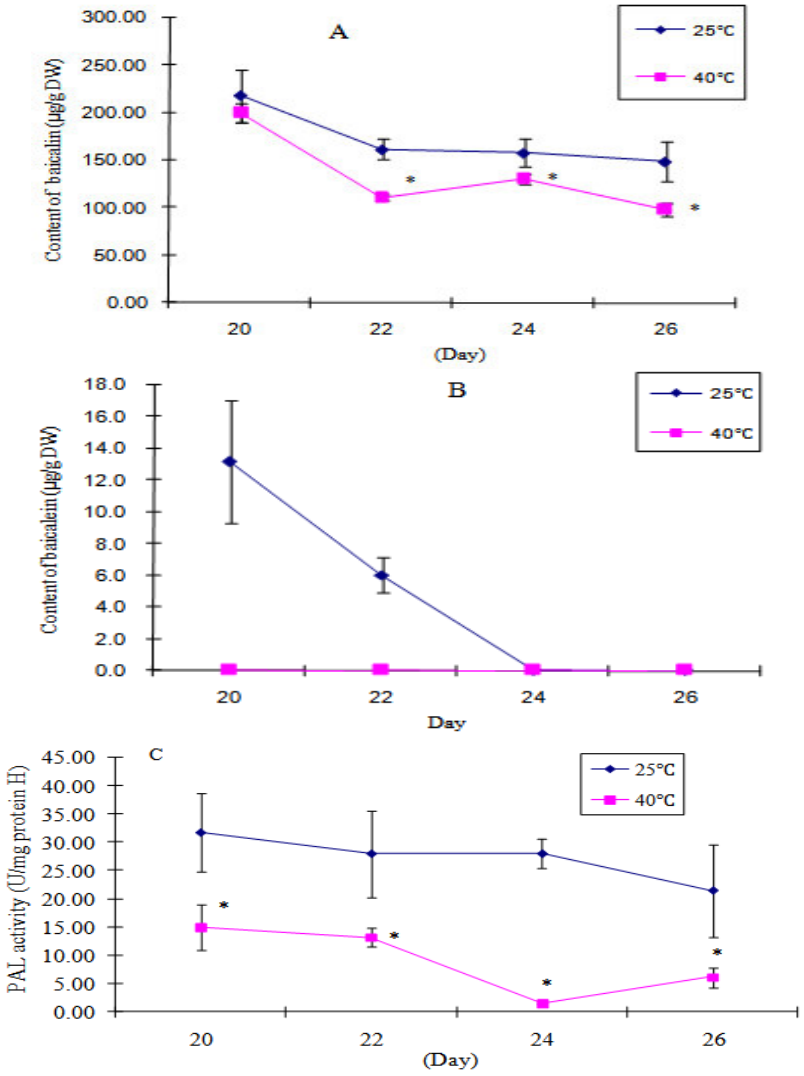

Figure 1. Contents of baicalin (A) and baicalein (B), and activity of PAL (C) in suspension cells after 20, 22, 24 and 26 days heat treatments. Each value represents the means ± SE of three replicates. * Denotes significant difference between 25 and 40℃ (t-test, $P < 0.05$).

volume was 20 μL for each sample solution and the experiment was repeated six times. The amounts of baicalin and baicalein were calculated following Li et al., (2009).

Total flavonoid assay

Total flavonoid content was quantitatively analyzed in the cell suspensions using the aluminum colorimetric assay (Marinova et al., 2005), calculated using a standard solution of baicalin. The test was repeated six times.

Statistical analysis

Variation was indicated by standard error (SE). Significant differences between treatments were calculated using *t*-tests. Correlation assay was performed with Excel 2003 (Microsoft) using the CORREL function.

RESULTS

Temperature effects on the flavonoid content and PAL activity

Baicalin and baicalein are major active compounds that accumulate in *S. baicalensis* and PAL is the first enzyme to produce baicalin in the phenylpropanoid pathway. To determine whether temperature affects flavonoid accumulation and PAL activity, we analyzed cells grown at 25 and 40℃. Baicalin and baicalein content and PAL activity decreased with the increasing duration of the growth period at both 25 and 40℃. Flavonoid content and PAL activity at 40℃ were lower than those at 25℃. Baicalein was undetectable in the cells grown at 40℃ (Figure 1). PAL activity was positively correlated with baicalin

content at both 40°C (R = 0.50) and 25°C (R = 0.80). Decreases in baicalin and baicalein content and PAL activity also occurred in samples moved to normal temperatures after heat stress treatment (Figure 2).

O_2^- production rate and H_2O_2 concentration

We measured O_2^- production rate and H_2O_2 concentration to determine whether high temperature causes oxidative stress in *S. baicalensis* cells. There were no significant differences in the O_2^- production rate or H_2O_2 concentration between 20 and 26 days of cultivation (Figure 3). O_2^- production rate and H_2O_2 concentration were negatively correlated with baicalin content at high temperatures and positively correlated with baicalin content at the control temperature. Cells moved to normal temperature after heat stress treatment had O_2^- production rates and H_2O_2 concentrations similar to those of the controls (data not shown).

Change in antioxidant enzyme activities

We analyzed SOD, CAT and POD activities to determine whether heat stress affects antioxidant enzymes in *S. baicalensis* cells. SOD activity after 24 days at 40°C was significantly lower than in the control. CAT activity at 40°C was significantly lower after 24 and 26 days in the culture compared to the control. Heat stress also led to decreased POD activity after 24 days in culture compared to the control (Figure 4). But increases in POD activity occurred in samples moved to normal temperatures after heat stress treatment (Figure 2) and after 22 days in culture compared to the control (Figure 4). Activities of antioxidant enzymes were negatively correlated with baicalin content in both high temperature (SOD, R = -0.93; CAT, R = -0.98; POD, R = -0.85) and in the control groups (SOD, R = -0.36; CAT, R = -0.86; POD, R = -0.52).

Changes in GUS activities

GUS catalyzes the formation of baicalin from baicalein and its activity may change under heat stress. GUS activity at 40°C was 43.5% lower after 22 days than in the control (Figure 5). GUS activity was negatively correlated with baicalin content in both high temperature (R = -0.61) and control groups (R = -0.37). Cells cultured under normal temperature following heat treatment stress had significantly higher GUS activity than the control (Figure 5).

Changes in cell suspension dry weight

We measured the dry weight of cell suspensions to determine whether high temperature affects the growth of *S. baicalensis* cells. As shown in Figure 6, after 20 and 26 days of culture, the dry weights at 40°C were 3.0 and 2.4% higher than those of the controls, respectively.

DISCUSSION

In general, metabolic reactions and growth increase with temperature, although high temperatures may cause cellular damage. At 40°C, the dry weights of the cell suspensions of *S. baicalensis* increased over 20 days of culture, indicating that high temperature did not affect cell growth. High temperatures may also impact the content of bioactive compounds. For example, high temperature leads to a lower content of kaempferol, a type of flavonoid, in broccoli (Mrkic et al., 2006) and the content of lyco-pene and β-carotene in tomato decline significantly at higher temperatures (Rosales et al., 2006). Pan et al. (2004) reported a progressive reduction in anthocyanin content of strawberry fruit at temperatures ranging from 30 to 45°C and they suggested that this explained the onset of oxidative damage. In *S. baicalensis*, baicalin and baicalein are major active compounds involved in the elimination of ROS. The metabolism of these compounds may determine adaptive responses to different types of stress. In this study, baicalin and baicalein content declined significantly in cells grown at 40°C (compared to the control), which is consistent with earlier reports (Mrkic et al., 2006). PAL is the first enzyme in the phenylpropanoid pathway to produce baicalin. High PAL expression levels often occur in parallel with high flavonoid concentrations (Lillo et al., 2008). Cold treatment induces flavonoid biosynthesis in apple (Ubi et al., 2006), maize (Christie et al., 1994) and red orange (Lo Piero, 2005). Decreases in flavonoid content might be due to decreases in synthesis or increases in degradation. In our study, long-term heat treatment led to a decrease in PAL activity, which was positively correlated with the content of active com-pounds, suggesting that high temperatures inhibited flavonoid biosynthesis.

There is an ROS burst when cells are first placed under stress conditions. In this study, long-term heat stress in *S. baicalensis* cells did not affect O_2^- production rate or H_2O_2 concentration, indicating that cells had recovered from early oxidative stress. Antioxidant systems in plants prevent or alleviate membrane peroxidation caused by ROS under high temperatures or other stressful conditions (Tasxgin et al., 2006). SOD and CAT constitute the suite of enzymatic defenses against oxidation stress (Rao et al., 1996).

CAT is the major enzyme responsible for eliminating H_2O_2 in mitochondria and microbodies and it is involved in the regulation of plant stress responses (Shigeoka et al., 2002). Stressors decrease CAT activity in plants. In dark-treated pepper leaves at high temperatures, loss of CAT activity may be a consequence of membrane

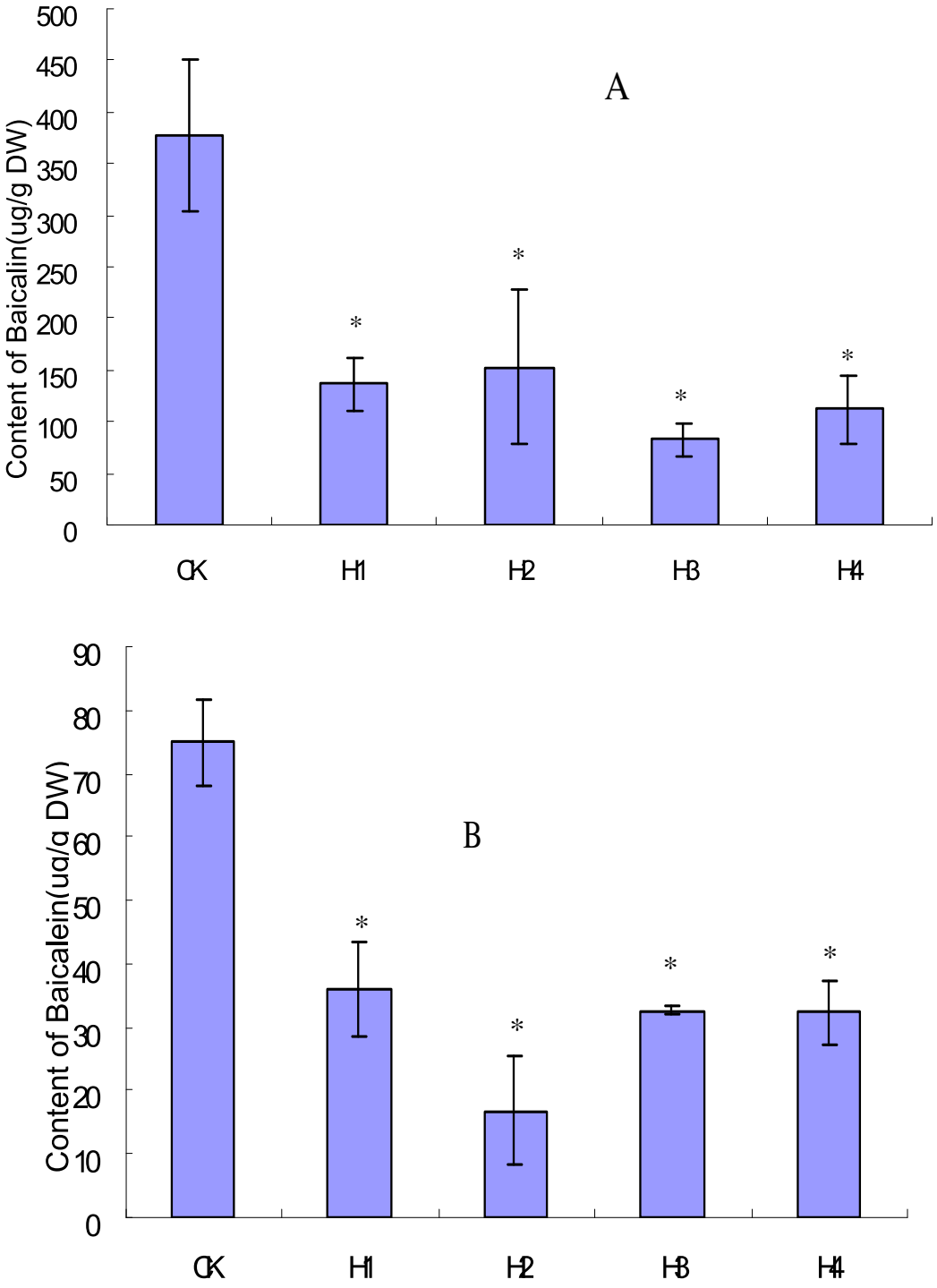

Figure 2. Contents of baicalin (A), baicalein (B), activity of PAL(C) and POD (D) in suspension cells recovered from certain time of heat stress. Each value represents the means ± SE of three replicates. *Significant difference between 25 and 40°C

dysfunction (Anderson, 2002); this may also be the case in chill-stressed cucumber leaves (Xu et al., 2008). At 40°C, CAT activity in cell suspensions of *S. baicalensis* declined after 24 days of culture, suggesting membrane dys-function at high temperature. SOD is located in chloroplasts, mitochondria, cytoplasm and peroxisomes, and acts as the first line of defense against ROS by dismutating O_2^- to H_2O_2 (Liau et al., 2007). In this study,

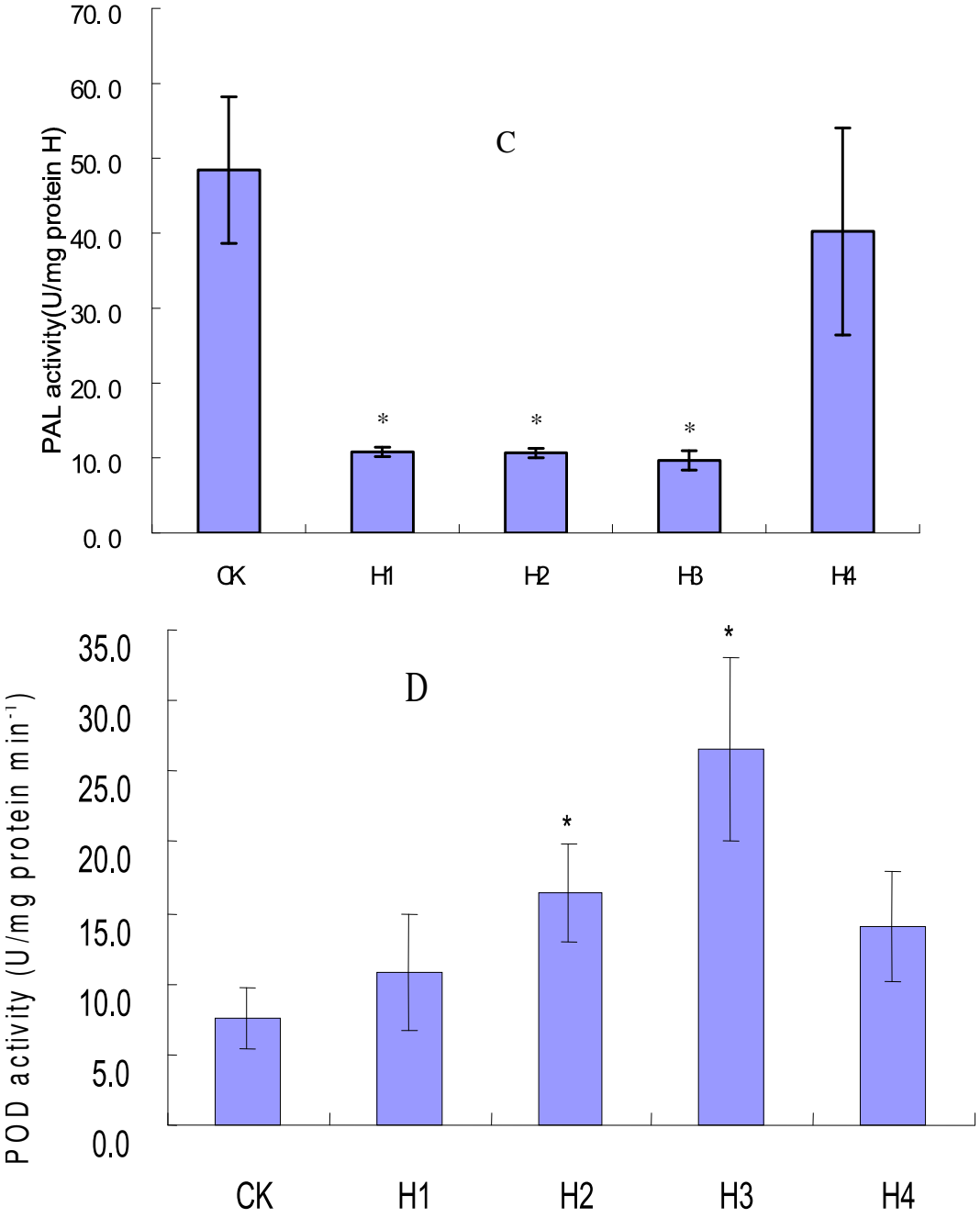

Figure 2. Continued

SOD activity also decreased after 24 days of culture. Lower CAT and SOD activities were linked to the levels of active compounds in cell suspensions, suggesting that the balance between ROS and antioxidant enzyme activities may be important in heat tolerance, rather than the absolute level of enzyme activity.

However, high temperatures increased POD and GUS activities in the cell suspensions after 22 days of culture. It is possible that a cell elicitor initiates hydrolysis of baicalin to baicalein (catalyzed by GUS) and the released baicalein was then quickly oxidized to 6,7-dehydro-baicalein while hydrogen peroxide (H_2O_2) was inactivated by POD. Hence, POD may be an important antioxidant enzyme that scavenges or utilizes H_2O_2 in *S. baicalensis* cells. This would imply that *S. baicalensis* cells with low flavonoid content or reduced transformation between types of flavonoids may use their antioxidant system to help tolerate long-term heat stress.

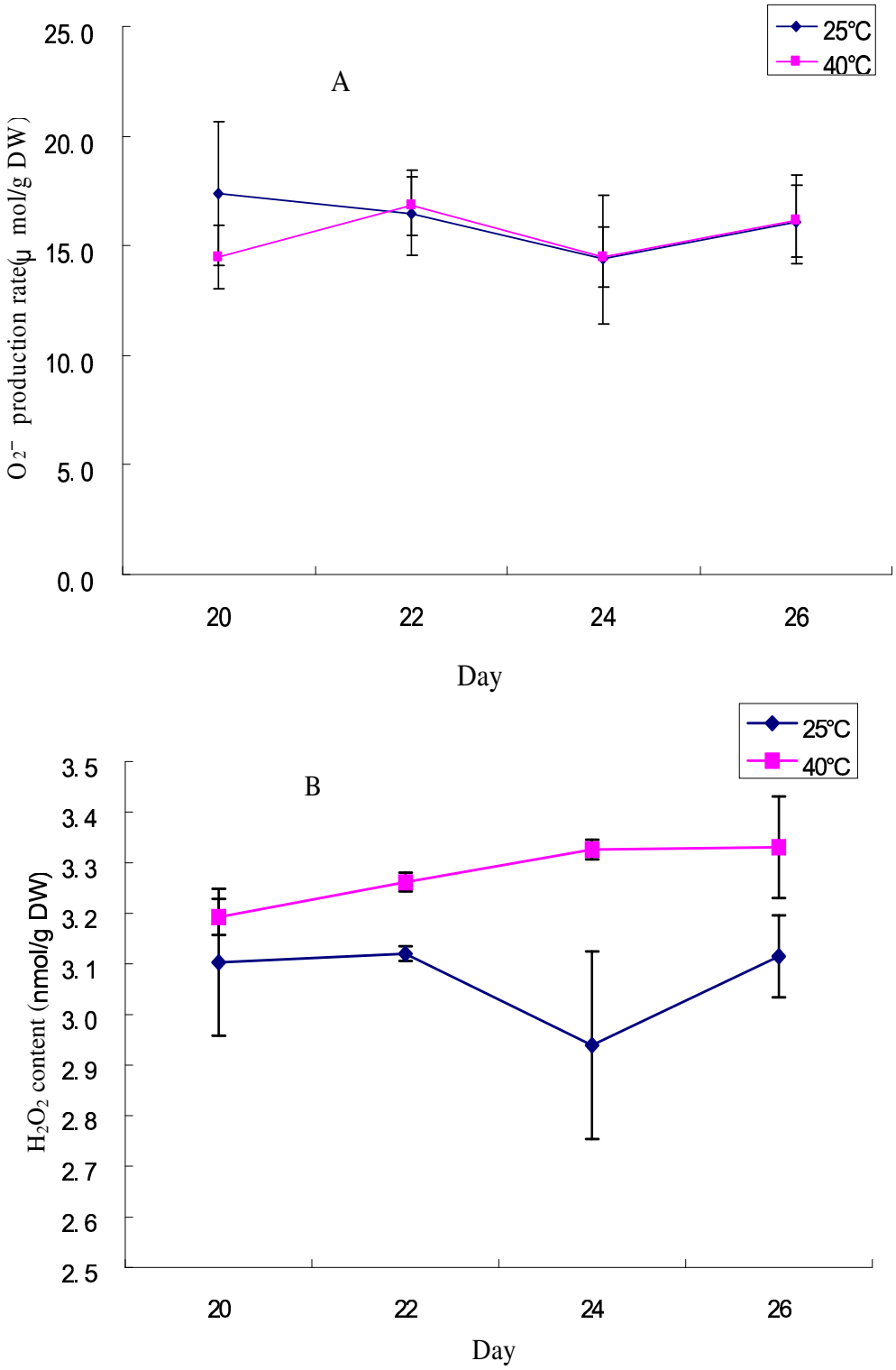

Figure 3. O_2^- production rate (A) and H_2O_2 concentration (B) in suspension cells after 20, 22, 24 and 26 days treatments. Each value represents the means ± SE of three replicates.

In conclusion, long-term exposure to high temperatures did not affect *S. baicalensis* cell growth but inhibited flavonoid biosynthesis and reduced the content of baicalin and baicalein. These two compounds play

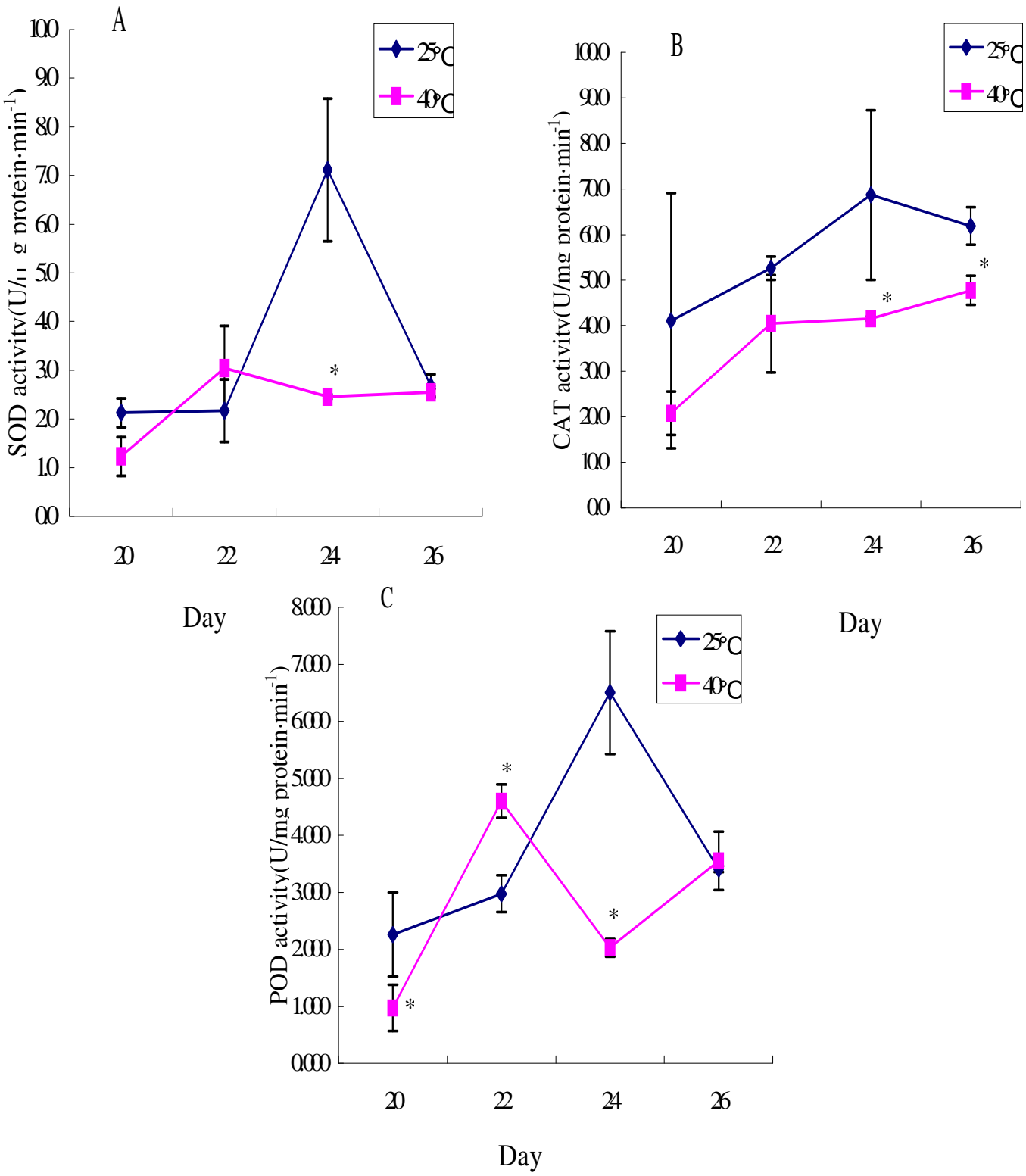

Figure 4. Activity of SOD (A), CAT (B) and POD (C) in suspension cells after 20, 22, 24 and 26 days treatments. Each value represents the means ± SE of three replicates. *Significant difference between 25 and 40°C (t-test, $P < 0.05$).

important roles in the balance between ROS and antioxidant enzyme activities in adaptive responses to high heat. This suggests that *S. baicalensis* may have genes that can be manipulated to improve other crops

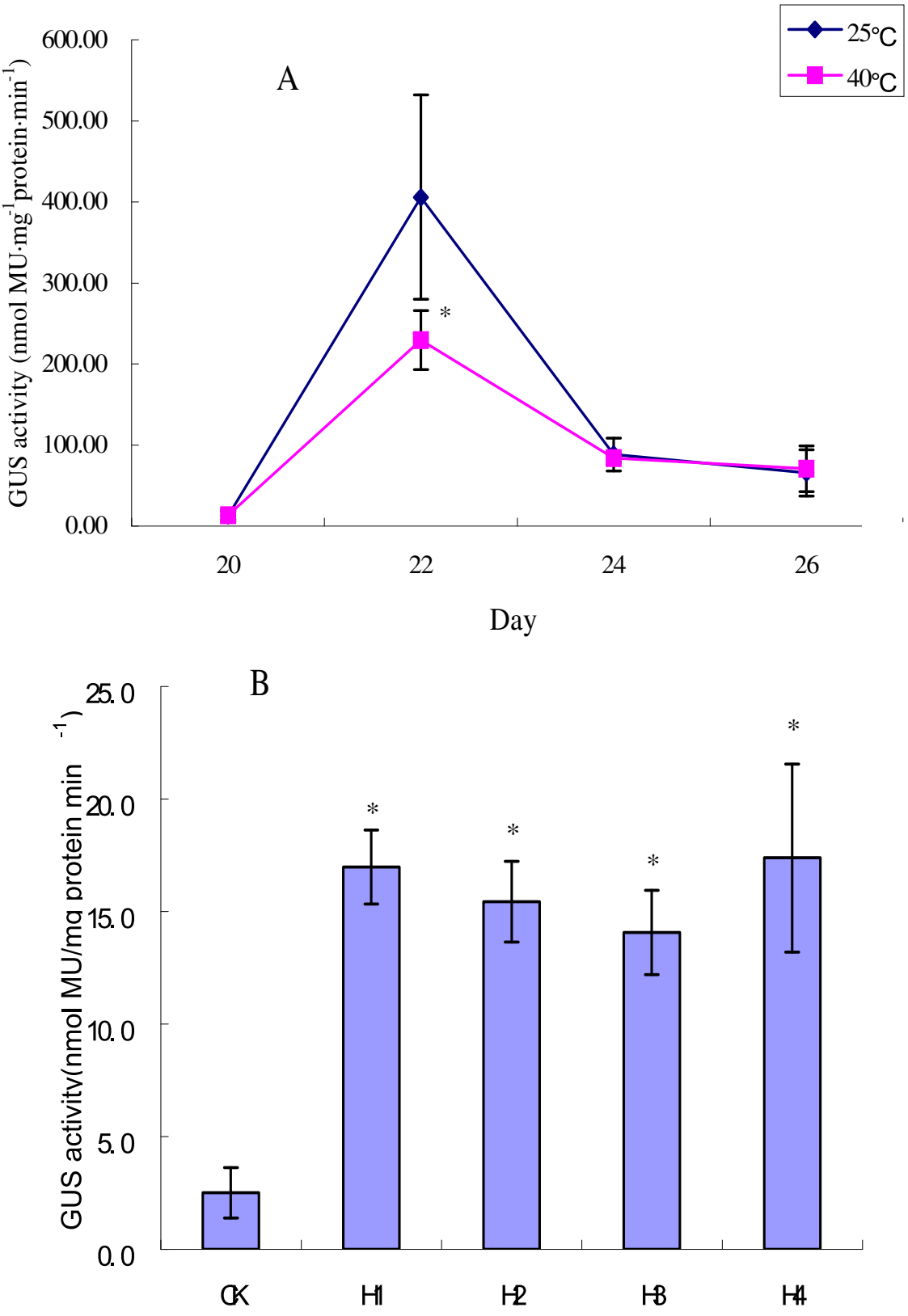

Figure 5. GUS activity in suspension cells after 20, 22, 24 and 26 days treatments (A) and in suspension cells recovered from certain time of heat stress (B). Each value represents the means ± SE of three replicates. * Denotes significant difference between 25 and 40°C (t-test, $P < 0.05$).

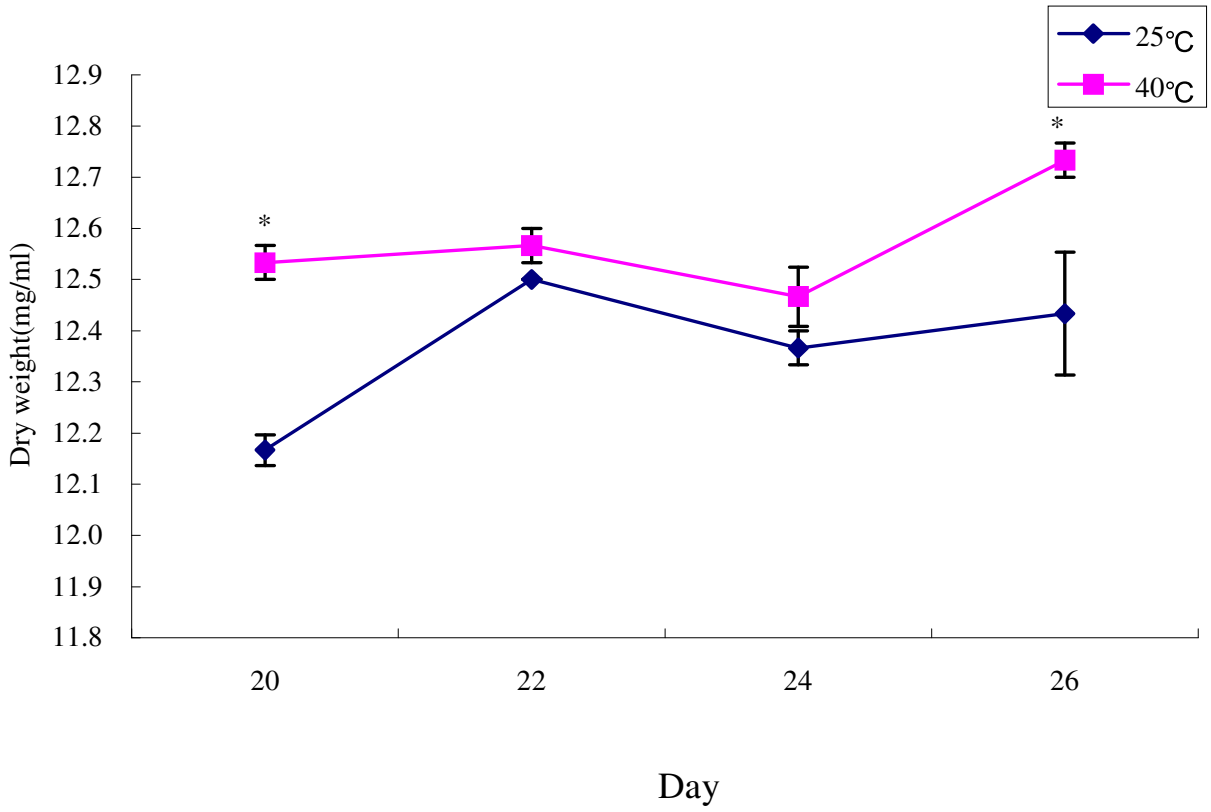

Figure 6. Dried weight of suspension cells after 20, 22, 24 and 26 days treatments. Each value represents the means ± SE of three replicates. * Denotes significant difference between 25 and 40℃ (t-test, $P < 0.05$).

adaption to high temperatures.

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