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ORIGINAL ARTICLE

High-temperature Condition Increases Lignanoid Biosynthesis of *Schisandra chinensis* Seeds via Reactive Oxygen Species

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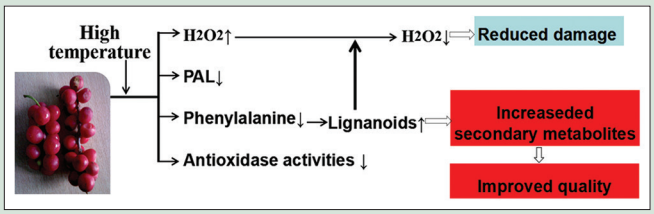
ABSTRACT

Introduction: The herbal medicine used in many countries came mainly from the wild in the past; now, declining yield resource and laborious gathering result in prevailing cultivated medicine, with a result of prevailing inferior quality of herbal medicine. The contents of major functional ingredients varies greatly in the fruits of *Schisandra chinensis*, a herbal medicine in many Asian countries. **Materials and Methods:** These fruits were placed at 20°C, 35°C, 45°C, and 55°C for 1–6 days, respectively, covered with plastics to prevent cells from anhydration during treating. The contents of H_2O_2, phenylalanine, and lignanoids and activities of antioxidant enzymes and phenylalanine ammonia lyase (PAL) were monitored. **Results:** The fresh seeds were exposed to 35°C, 45°C, and 55°C for 1 week; the H_2O_2 was rose sharply at 1 day and then declined but still with a higher level. The superoxide dismutase, catalase, and peroxidase activities were lowered, with inefficient antioxidant capacity. The PAL activities had a certain degree of high-temperature tolerance, remained largely unchanged at 35°C, but reduced gradually as temperature increased. High temperature activated the glycolytic pathway and rose the phenylalanine contents, which increased sharply at 1 day for 35°C and 45°C and at the 2 days for the 55°C and then maintained a stable level with almost 1–3 times than the 0 day. **Conclusions:** The increased phenylalanine as substrate accelerated the synthesis of lignanoids; the contents of five lignanoids were increased by as much as 31.2%–81.5%, respectively.

Key words: Environmental stress, lignanoids, phenylalanine, *Schisandra chinensis*, secondary metabolism

SUMMARY

- The abscised fresh fruits of *Schisandra chinensis* are live organisms and remain intrinsic total metabolic system. The fresh fruits were exposed to high-temperature condition, the antioxidase was lowered, the phenylalanine ammonia lyase activities also did gradually as temperature increased, but the glycolytic pathway was activated, and the phenylalanine contents were increased, enhancing the synthesis of lignanoids. "↑" and "↓" represent activity or content "up" and "down," respectively.



Abbreviations Used: ROS: Reactive oxygen species; SOD: Superoxide dismutase; CAT: Catalase; POD: Peroxidase; PAL: Phenylalanine ammonia lyase.

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INTRODUCTION

The herbal medicine used in many countries came mainly from the wild in the past; now, declining yield resource and laborious gathering result in prevailing cultivated medicine. The major functional ingredients of herbal medicine, usually the secondary metabolites, are to prevent plant from stress environment; therefore, its contents varies depending on the environment, with a result of prevailing inferior quality of herbal medicine, even have a word, "traditional Chinese medicine would come down," "the traditional Chinese medicine would be destroyed by herbal medicine." China has used the herbal medicine for several years; the conversion of herbal medicine from wild to cultivation threatened to the survival of traditional Chinese medicine. The Chinese Government promulgated "Good Agriculture Practice for Chinese crude drugs" to control various factors affecting the production quality of medicinal plant materials and further to ensure that traditional Chinese medicine herbs are authentic, safe, effective, and consistent in quality.[1] Besides China, many countries have taken a series of standardized measures concerning quality control of production of raw materials for natural medicines,[2] but all these pay attention to production procedure, not to specific active ingredient relevant to the effect, with a little success. How to improve the quality of herbal medicine is emphasis and difficulty.[3] The clarification of herbal medicine ingredients' biosynthesis may be a reasonable way to improve cultivated herbal medicine.

Different species have a highly specific trait to acclimate various environment; even the different organs or tissues of the same species may vary significantly.[4] *Schisandra chinensis* (Turcz.) Baill. is a deciduous, perennial vine from *Magnoliaceae*, habitat in the bushes. Its fruits ripen in the autumn, but the seeds, still in the heart-shaped embryo stage, will take another 3 months to further grow and develop after abscission[5] and have to undergo frequently various stresses such as high temperature, drought, and microbes, during germination. A simple change during this critical life stage may threaten germination.[6] Therefore, *S. chinensis* is a better species to investigate into seeds against the environment.

There were two primary sides for plant damage by stress: physical and mechanical injury and chemical injury. The physical and mechanical injury derives mainly from the low temperature, pests, etc., whereas

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the chemical injury mainly from the high temperature, water deficit, saline-alkali soil, environmental contamination, etc., Animals can dodge dreadful conditions, but plants must be disturbed by various environmental stresses. One of the major consequences of stress is the excess generation of reactive oxygen species (ROS), with a result of oxidative stress.[7,8] For this reason, the plant develops a exclusively secondary metabolism pathway to combating the stress. The secondary metabolites, responsible for the enhanced stress tolerance,[9] are produced by induction of ROS. The lignanoids in *S. chinensis* fruit, such as schizandrol A, schizandrol B, shiandrin A, shiandrin B, and shiandrin C, with neuroregulation, liver protection, antioxidation, and anti-allergic effects,[10] are used as medicine in most Asian countries.[11] Therefore, an exhaustive research into the secondary metabolites has been done. The previous study showed the secondary metabolites of *S. chinensis* fruit are able to detoxify ROS,[12,13] biosynthesized by phenylalanine ammonia lyase (PAL). PAL, a typical of inducible enzymes, can respond to various unfavorable circumstance quickly;[14] even within 10 min under 40°C, the hypericin biosynthesis in *Hypericum perforatum* L. cells can be enhanced.[15] Thus, the phenylpropanoid metabolic pathway is far more susceptible to circumstance than any other pathways. The lignanoids relate to the temperature and rainfall,[16] which vary greatly between producing areas.[17-20]

Plants are constantly challenged by various ever-changing abiotic stresses under which plants generate and accumulate ROS.[21] The stresses are various, and their essence of influencing on plants are all ROS.[22] One of them is H_2O_2, which plays a major role in tolerance.[23] Earlier research has found some interesting facts that plants possess cross-resistance, meaning that one stress can result in increased tolerance of another stresses. Heat hock not only raises heat resistance but also induces tolerance to chilling, drought, and salinity and heavy metal in different plant species.[24-27] High-temperature stress can result in cellular damage and even cell death.[21] At germination, seeds tolerate various environmental stresses such as drought, high temperature, and microbes, but the high temperature was manipulated easier. Plants can produce ROS during high-temperature stresses;[21] therefore, exposure of *S. chinensis* seeds can easily promote the biosynthesis of lignanoids and provide an avenue for improved herbal medicine quality.

MATERIALS AND METHODS

Medicinal material collection and treatment

Fresh fruits of *S. chinensis* were collected, on September 9, 2015, from the medicinal garden of Heilongjiang University of Chinese Medicine, China, homogenized. These fruits were divided into 20°C, 35°C, 45°C, and 55°C groups, each group into six parts, placed in thermostatic drying chamber for 1, 2, 3, 4, 5, and 6 days, respectively, and covered with plastics to prevent cells from anhydration during treating.

Determination of H_2O_2 content

H_2O_2 was determined using a plant H_2O_2 ELISA kit that was purchased from Shanghai Yu Ping Biotechnology Limited Company, China.

Determination of enzyme activities

Superoxide dismutase (SOD) activity (U), assayed based on the reduction of nitroblue tetrazolium (NBT), was defined as the activity of enzyme that caused 50% inhibition of NBT reduction.[28] Catalase (CAT) activity, monitoring the decrease of H_2O_2 at 240 nm for 1 min at 25°C, was calculated as the activity of enzyme that caused a reduction in absorbance at 240 nm of 0.01 per min.[29] Peroxidase (POD) activity, determined the absorbance changes at 470 nm and 25°C, was defined as the activity of enzyme that caused an increase in absorbance at 470 nm of 0.001 per min.[30]

PAL activity was determined as per the method of Hussain *et al.*[31] A total of 1.0 g of fresh seeds was ground in an ice bath with 10 ml 0.1 mol/L boric acid buffer solution (pH 8.8) containing 1.0 mmol/L EDTA, 5% glycerol, and 5% polyvinylpyrrolidone; the extracts were centrifuged at −4°C 10000 r/min for 20 min. Added above-mentioned 1.0 ml supernatant and 2.0 ml 0.1 mol/L boric acid buffer solution, then added 0.01 ml H_2O, and 0.01 ml 0.4 mol/L H_2O_2 respectively. They were placed in 30°C water, 1 h later, inactivated with 0.2 ml 6 mol/L HCl, and centrifuged at 130,00 rpm for 15 min, and the change of 0.01 optical density values, measured at 290 nm by the spectrophotometer, was defined as one unit of enzyme activity.

$$\text{PAL}\left(\text{U}/\text{g}\bullet\text{h}\right)=\frac{\text{A}_{290}\times\text{ extract volume}\times\text{system volume}}{\text{Root weight }\times\text{ enzymes volume }\times 0.01\times\text{ duration}}$$

The above-mentioned sample was determined at the same temperature as it was placed.

Determination of phenylalanine

A volume of 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 ml of 40 μg/ml phenylalanine standards were added to test tubes, respectively; distillation water was added to 4.0 ml; afterward, 1 ml of 1.2% ninhydrin aqueous solution and 1 ml of pH 8.04 phosphate buffer solution were added to each of seven tubes, respectively, and blended. The reaction was performed in a water bath at a constant temperature of 90°C for 25 min, then cooled down, added loss, blended, and left to stand for 15 min. The absorbance at 570 nm was determined using ultraviolet spectrophotometry, drew the standard curves.

A total of 1.0 g fresh seeds was ground in 0.5 ml 10% ethylic acid solution, converted into 10 ml volumetric flask, water-volumed. The obtained solutions were centrifuged at 13,000 rpm for 20 min, and the absorbance of supernatant was determined. The phenylalanine was calculated as follows:

$$\text{Phenylalanine}\left(\text{mg}/\text{g}\right)=\frac{\text{Phenylalanine}\left(\mu\text{g}\right)\text{ in standard curves}}{\text{Seeds}\left(\text{g}\right)\text{ in determined solution}\times 1000}$$

Phenylalanine origin

H_2O_2 was determined using 1,3-diphosphoglyceric acid ELISA kit that was purchased from Shanghai Fu Sheng Biotechnology Limited Company, China.

The 1.0 g seeds of 20°C and 45°C groups were ground into homogenates with an appropriate amount of saturated $(NH_4)_2SO_4$; then,

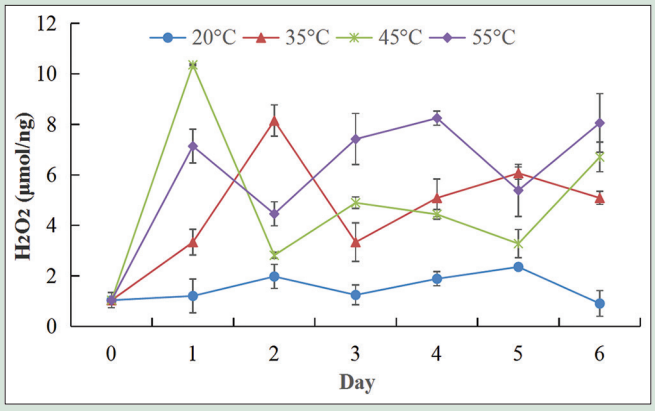

Figure 1: Mount of H_2O_2 changed under high temperature. The 0 day and 20°C had not altered the H_2O_2, the high temperature increased the H_2O_2 remarkably, with a tendency of increases first, then decreases, but still with a higher level than the 0 day. Among them, the 55°C had the highest contents

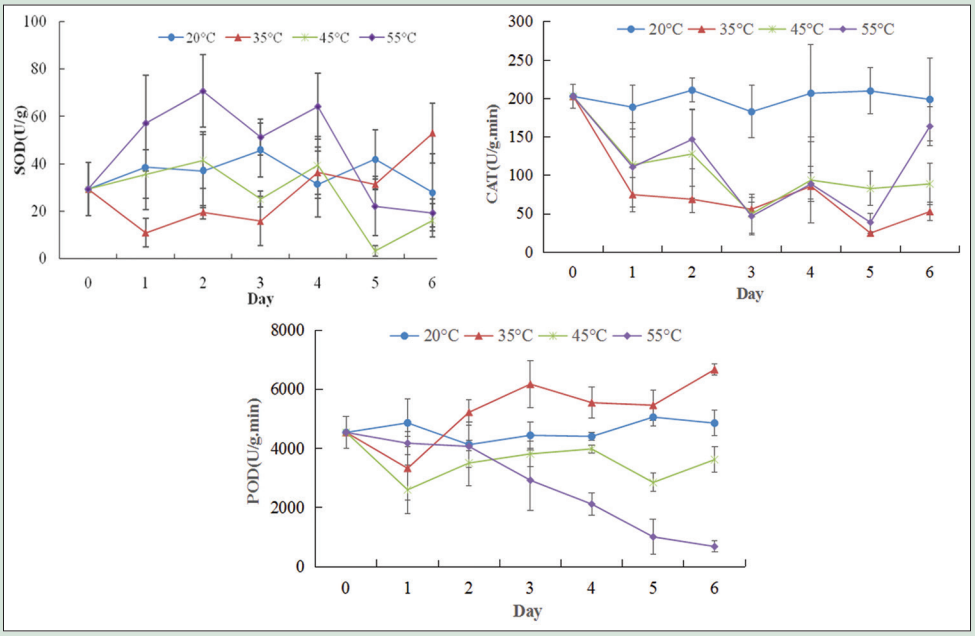

Figure 2: Effect of high temperature on the antioxidant activities. The SOD rose at the 55°C and lowered at 35°C; the POD was the opposite and was changed little at the 45°C. The CAT activities of the 35°C, 45°C, and 55°C had been remarkably reduced in all stages, indicating that the antioxidant action was lowered than the 0 day and the 20°C. SOD: Superoxide dismutase; CAT: Catalase; POD: Peroxidase

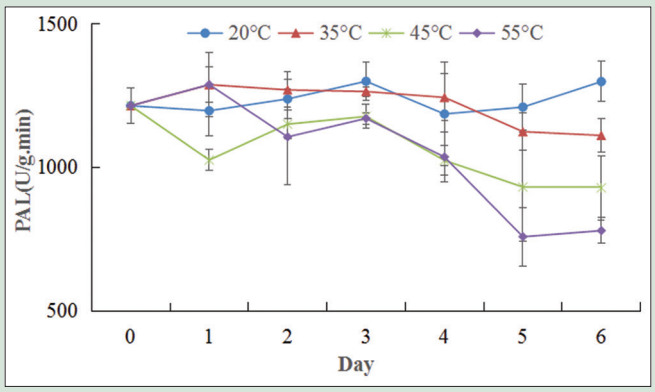

Figure 3: Change of the PAL activities. The PAL had a certain degree of resisting high temperature, remained largely unchanged at 20°C and 35°C, but reduced gradually as temperature increased, 35°C > 45°C > 55°C, indicating that the PAL did not work very well at all. PAL: Phenylalanine ammonia lyase

saturated $(NH_4)_2SO_4$ was added to 10 ml and centrifuged at 13,000 rpm for 20 min. The liquid supernatant was used for the determination of 1,3-diphosphoglyceric acid.

The sediments were washed with saturated $(NH_4)_2SO_4$ twice and re-dissolved with pH 8.04 phosphate buffer, and the crude enzymes were obtained. Four test tubes were all numbered; the number 1 (for 20°C) and the number 2 (for 45°C) were added 1 ml liquid supernatant and 1 ml 0.16 nmol/ml glucose, while the number 3 (for 20°C) and the number 4 (for 45°C) were added 1 ml liquid supernatant and 1 ml soybean protein solution. They all were placed at 30°C for 2 h and then determined the contents of L-phenylalanine.

Above all solution and utensils were cooled to 4°C below during extracting. The procedure was repeated three times.

Determination of lignanoids

0.25 g of the dry *S. chinensis* fruit power (day <0.1 mm) was put in a 25 ml volumetric flask; then, ultrasonic extraction with 70% methanol for 30 min; finally, the supernatant was filtered with a 0.22-μm microporous filter for ultra-high performance liquid chromatography analysis.

The experimental samples were analyzed by a Waters ACQUITY HPLC. The trial samples were based on a BEH C_{18} column (2.1 mm × 50 mm, 1.7 μm). The mobile phases were composed of (A) H_2O with 0.5% glacial acetic acid and (B) methanol. The gradient program as initial 20% B ~ 60% B from 0 to 10 min, 60% B from 10 to 30 min, 60% B ~ 70% B from 30 to 50 min, 70% B ~ 80% B from 50 to 60 min, 80% B for 60 ~ 70 min, 80% B ~ 100% B from 70 to 75 min, 100% B for 75 ~ 85 min, 100% B ~ 20% B from 85 to 86 min, and 20% B for 86 ~ 96 min. The flow rate was set at 1 ml/min, and the column temperature was set at 30°C. The detection wavelengths of schizandrol A, schizandrol B, shiandrin A, shiandrin B, and shiandrin C were 250 nm.

Data analysis

All the experimental data were analyzed using Excel (Microsoft Corp) and expressed as the mean ± standard error of the mean. The 35°C, 45°C, and 55°C were chosen as high-temperature condition; the 20°C was chosen as nonstress condition. The high-temperature effect was determined by comparison with the 0 day.

RESULTS

H_2O_2 contents

The amount of H_2O_2 was increased sharply in fresh fruit under 35°C, 45°C, and 55°C [Figure 1]. The 45°C and 55°C peaked at 1 day and the 45°C at 2 days; then, they all declined but still with a higher level than the 0 day. Among them, the 45°C had the sharpest rise, and the 55°C had the highest contents.

Antioxidant activities

The high temperature had various effects on the SOD activities, rose at the 55°C, lowered at 35°C, and changed little at the 45°C. The CAT activities of the 35°C, 45°C, and 55°C had been reduced in all stages. All the POD activities had been reduced only at the 1 day, then rose at the 35°C, lowered at 55°C, and changed little at the 45°C [Figure 2]. Altogether, the antioxidant action was lowered than the 0 day and the 20°C.

Phenylalanine ammonia lyase activities

The PAL activities had a certain degree of resisting high temperature, remained largely unchanged at 35°C, but reduced gradually as temperature increased, 35°C > 45°C > 55°C [Figure 3]. Varying high temperature rose the phenylalanine contents, increased sharply at the 1 day for the 35°C and 45°C and at the 2 days for the 55°C and 45°C, and then maintained a stable level with almost 1–3 times than the 0 day and the 20°C [Figure 4].

Phenylalanine origin

The 1,3-diphosphoglyceric acid content of the 45°C was higher remarkably than that of the 20°C ($P \leq 0.01$). For the glucose and protein group, the phenylalanine content from 45°C seeds were all higher than that of 20°C, but the statistically significant differences exist only in glucose group [Figure 5].

Lignanoid contents

Varying high temperature rose the contents of schizandrol A, schizandrol B, shiandrinA, shiandrin B, and shiandrin C, with similar trends. The contents increased gradually as time goes on. At 6 days, the contents of schizandrol A, schizandrol B, shiandrin A, shiandrin B, and shiandrin C increased by 48.0%, 64.6%, 81.5%, 56.8%, and 31.2%, respectively, than the 0 day and the 20°C [Figure 6].

DISCUSSION

Plants can produce ROS during high-temperature stresses.[21] The several locations that the chlorophyllous tissues produce ROS are mainly in chloroplasts and mitochondria and the other organs mainly in mitochondria, plasma membranes, peroxisomes, apoplast, endoplasmic reticulum, and cell walls.[32] The most common ROS in the nonchlorophyllous tissues include $O^{\bullet -}_2$, H_2O_2, and $^{\bullet}OH$. Activation of O_2 occurs mainly by stepwise monovalent reduction; O_2 is sequentially reduced to $O^{\bullet -}_2$, H_2O_2, and $^{\bullet}OH$. H_2O_2 has a half-life of only 1 ms and is relatively stable as compared to $O^{\bullet -}_2$ and $^{\bullet}OH$ that have only 1 μs and 1 ns, respectively.[33] Therefore, the contents of H_2O_2 generally present the level of whole ROS. Cell damage under stress condition results from ROS, and high level of ROS can modify adjacent molecular configuration, lower stability of lipid bilayer, and cross-link sulfur protein, with great damage to cell.[34]

Contents of H_2O_2 in *S. chinensis* seeds under high-temperature condition increased rapidly at the 1 day and then decreased [Figure 1]. The high-temperature condition as a fountain of H_2O_2 was continuous, the decrease of H_2O_2 was a dissonant dilemma, and the reason that decreased of H_2O_2 probably was the result of antioxidant. Plants possess complex antioxidative defense system, comprising antioxidase, secondary metabolites, glutathione, Vitamin C, *et al.*, to scavenge ROS. The antioxidants play an important role in scavenging ROS. The SOD of the 35°C and 45°C failed to increase, the CAT and the POD of the 45°C and 55°C did also, probably due to the damages of ROS to the antioxidase, a bioactive protein. The SOD cannot scavenge ROS without the help of CAT and POD *et al.*, the POD has a typically slowly reductive step, the CAT does not require cellular reducing equivalent and has a very fast turnover rate,[35] and the CAT therefore does excellent in scavenging ROS. These, in general, manifested that the antioxidant capacities were not increased but decreased [Figure 2], in spite of increase of SOD under 55°C and POD under 35°C. In this case, the ROS from high temperature were scavenged mainly by secondary metabolites.

Plants developed negative feedback self-regulation to eliminate ROS during the long-term evolution. Once the plants are subject to stresses, then ROS are rose and convert into more H_2O_2. The long-lived H_2O_2 as messenger of regulating metabolism acts as a central player in

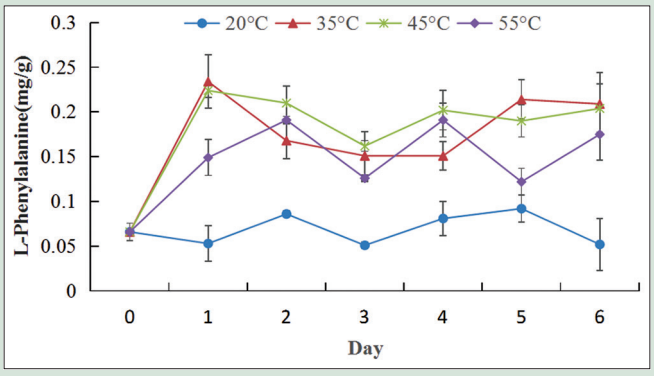

Figure 4: Change of the phenylalanine contents. Varying high temperature rises the phenylalanine contents, increased sharply, then declined, but still with as much as 1–3 times higher than the 0 day and the 20°C, which stimulated the synthesis of lignanoids

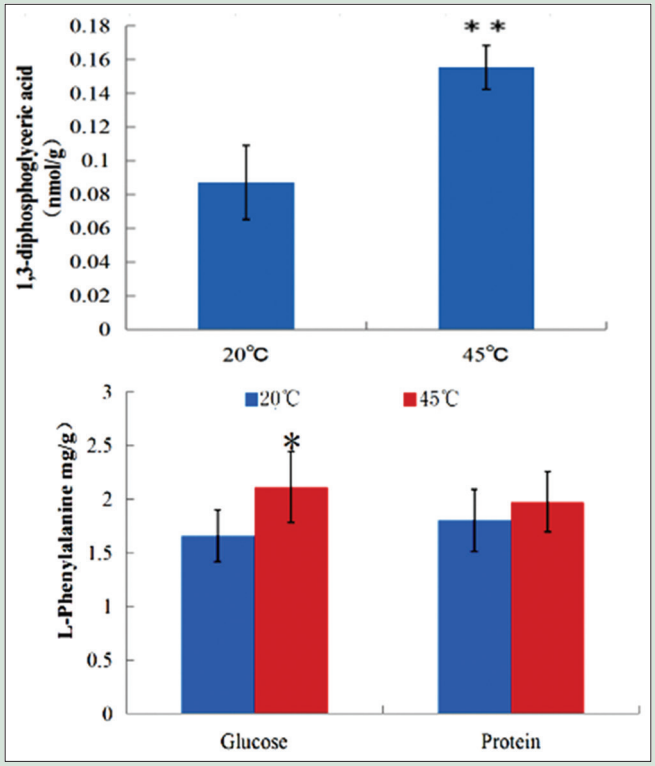

Figure 5: Origin of phenylalanine. The 1,3-diphosphoglyceric acid content of the 45°C was higher remarkably than that of the 20°C ($P < 0.05$)* ($P < 0.01$)**. For the glucose and protein group, the phenylalanine content from 45°C seeds were all higher than that of 20°C, but the statistically significant differences exist only in glucose group, indicating phenylalanine derives mainly from the degradation of glucose under high-temperature condition

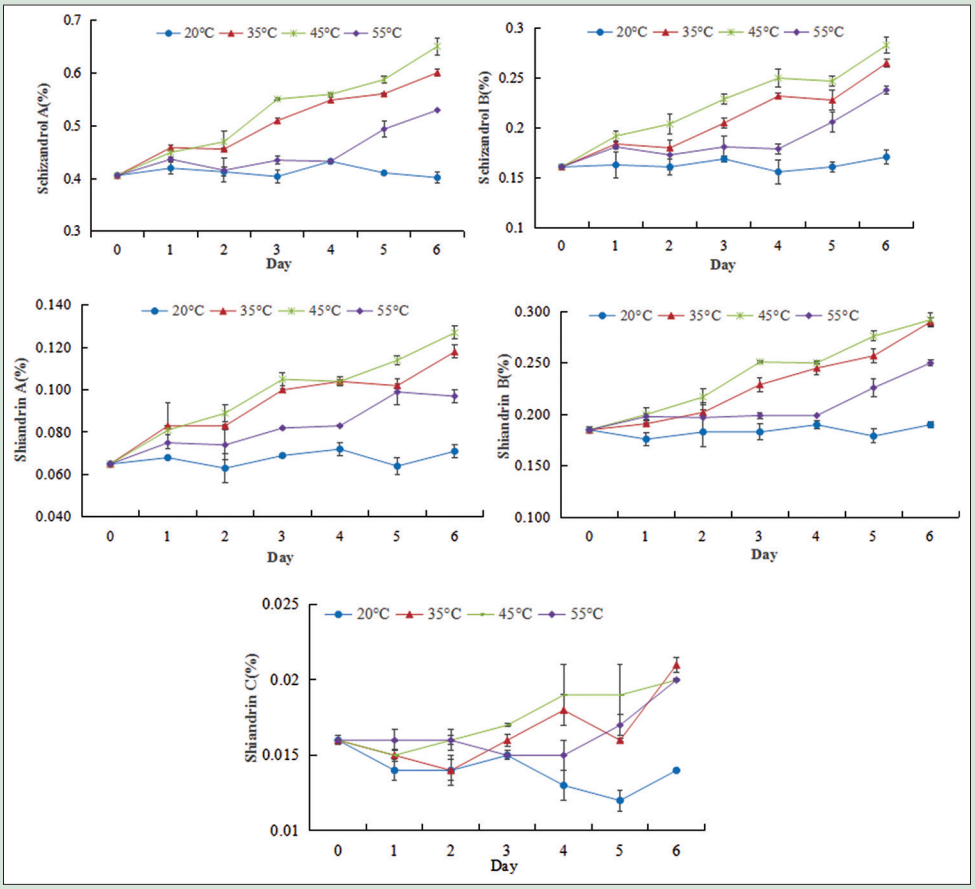

Figure 6: Change of the lignanoid contents. High temperature rises the contents of schizandrol A, schizandrol B, shiandrin A, shiandrin B, and shiandrin C, with similar trends. The contents increased gradually as time goes on. At 6 days, the contents of schisandrin, deoxyschizandrin, γ-shiandrin B, and shiandrin C increased by as much as 48.0%, 64.6%, 81.5%, 56.8%, and 31.2%, respectively, than the 0 day

stress signal transduction pathways. These pathways can then activate multiple acclamatory responses that reinforce resistance to various stresses.[23] With this, the secondary metabolism is upregulated by ROS,[33] and in turn, the increased secondary metabolites scavenge the redundant ROS.[36]

Biphenyl cyclooctene lignanoids are a class of chemical constituents with the capacity to eliminate ROS. PAL is a key enzyme for biosynthesis of lignanoids, whose optimal temperature is 30°C–65°C, higher than other enzymes. The PAL is a stress enzyme, used as a physiological marker for measuring the resistance of plants due to its strongly expressed under stresses.[37] Figure 3 shows that PAL had powerful resistance to high temperature. It was interesting that the PAL activities did not be increased but not decreased under high-temperature condition, while the contents of phenylalanine rose sharply and kept a high level [Figure 4]. The glycolytic pathway or the degradation of protein can be a major potential source of stress. The 1,3-diphosphoglyceric acid is one of the intermediates in glycolytic pathway. The increased 1,3-diphosphoglyceric acid revealed that the phenylalanine derives in the main from the degradation of glucose [Figure 5], which is sufficient in any tissue. High temperature can lead to protein degradation,[38] the phenylalanine can also result from the degradation of protein [Figure 5], but the mount may seem fairly trifling, maybe due to small amounts of phenylalanine in protein. Heat stress causes alterations in expression of genes involved in direct protection from high-temperature stress.[39] The biphenyl cyclooctene lignanoids are synthesized from phenylalanine; the increased phenylalanine stimulates lignanoids' synthesis. The exposure of the fresh fruit to 35°C, 45°C, and 55°C condition boosted the lignanoids, going on with time [Figure 5]. Under 55°C, both the PAL and the produced phenylalanine were the least, with a result of the least contents of total lignanoids, in this case more and more H_2O_2 probably damage the cells. Under 45°C conditions, although PAL activity was lower than the 35°C, the phenylalanine was the highest [Figure 4]; furthermore, SOD, CAT, and POD rose [Figure 3] and eliminated a great amount ROS, causing retention of more lignanoids than the 35°C.

CONCLUSIONS

Seeds as the pivotal organ for plant contain large quantities of stored carbohydrate, protein, and other reserves. The synthesis of lignanoids took advantage of phenylalanine from degraded glucose under high-temperature condition; the effectiveness of *S. chinensis* seeds could be improved heavily.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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