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Research Article
Effects of Drought Stress on Anthocyanin Accumulation in Mulberry Fruits

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Abstract

Background and Objective: Mulberry (*Morus* spp.) fruits are rich in anthocyanins which have diverse pollination and stress functions. Drought is an important environmental factor that adversely affects plant growth and productivity. However, its effect on anthocyanin response in biosynthetic pathways has not been studied in mulberry. This study aimed to investigate the anthocyanin accumulation and expressions of the relevant genes in mulberry plants exposed to drought. **Materials and Methods:** The plants were exposed to simulated drought by withholding water for 0, 5, 7 or 10 days. The levels of anthocyanins were determined using high-performance liquid chromatography. Gene expression was analyzed by quantitative real-time PCR. **Results:** Cyanidin-3-glucoside and malvidin-3-glucoside contents notably increased after 10 days of drought. High levels of Phenylalanine Ammonia Lyase (*PAL*), Chalcone Synthase (*CHS*) and Anthocyanidin Synthase (*ANS*) were detected in the fruits in response to drought. The fruit extracts had DPPH-scavenging and DNA damage protection activities, which could be used in disease prevention. In addition, there was a positive correlation between the increased anthocyanidin content during drought and antioxidant activity. **Conclusion:** Drought stress significantly enhanced anthocyanin production, possibly due to changes in anthocyanin biosynthesis gene expressions, thus, drought stimulation is a potential method to develop anthocyanin-rich mulberry fruit for the pharmaceutical, food and cosmetic industries.

Key words: Anthocyanin, drought, mulberry, HPLC, gene expression

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The mulberry is a dicotyledon belonging to the family Moraceae, genus *Morus*; it has multiple uses including silkworm rearing, ecology, pharmaceuticals and traditional Chinese medicines[1]. There are various mulberry species and varieties widely distributed in Asia, Europe, Africa and North and South America. In Thailand, mulberry plants are widely cultivated, especially in the northeastern region. Mulberry fruits have been known as a rich source of bioactive compounds including anthocyanins, flavonols, phenolic acids and amino acids[1]. Several studies have demonstrated the biological activities of mulberry fruits, such as antimicrobial[2], anti-inflammatory[3] and antioxidant[4] activities.

Anthocyanins are water-soluble pigments that play diverse roles in plant development and in environmental interaction[5,6]. In addition to their colorful characteristics, anthocyanins protect plants from several biotic and abiotic stresses[7,8], which may provide them with better adaptation to climate change. Anthocyanins are of considerable interest to researchers due to their powerful antioxidant properties. Anthocyanins are synthesized from the flavonoid biosynthetic pathway derived from the general phenylpropanoid pathway, one of the important pathways in plant secondary metabolism. Flavonoids play important roles in plants, such as pigmentation, plant reproduction by recruiting pollinators and seed dispersers and by stress protection[9]. These compounds have also been suggested to be antioxidants due to their hydroxyl groups, double carbon bonds and glycosylation[10]. Several enzymes are involved in anthocyanin biosynthesis such as Phenylalanine Ammonia Lyase (*PAL*), Chalcone Synthase (*CHS*), Chalcone Isomerase (CHI) and Anthocyanin Synthase (*ANS*)[11]. The most common anthocyanins found in plants are the derivatives of six widespread anthocyanidins, namely, cyanidin, delphinidin, pelargonidin, peonidin, petunidin and malvidin[12].

Plants frequently encounter unfavorable growth conditions throughout their life cycles. Climatic factors, such as extreme temperature, high salinity and drought are important abiotic stresses that affect plant growth and productivity[13]. Prolonged drought reduces soil water content and leads to water-deficit stress conditions. Mulberry adapts well to drought, salinity, waterlogging and other abiotic stress conditions, however, little is known regarding the molecular mechanisms of these tolerances. Several environmental stresses induce anthocyanin biosynthesis such as strong light and low temperature[7,14,15]. For example, increased anthocyanin levels play an important role in protecting the leaves from photodamage caused by stress from strong light[16]. Low temperature significantly induces anthocyanin accumulation[17,18], whereas high temperature leads to a reduction of anthocyanin content in apple[19]. The present study investigated the effects of drought stress on anthocyanin accumulation in Thai mulberry.

MATERIALS AND METHODS

Study area: This study was conducted at the Faculty of Science, Kasetsart University, Thailand, from November, 2016 to October, 2020 period.

Plant materials and growth conditions: Mulberry plants cv. Kamphaeng Saen-MB-42-1 were grown in a greenhouse under normal cultivation practices before initiating drought treatments. Different tissues were collected from plant leaves, inflorescence and fruits at three different fruit stages, namely, green fruit, orange fruit and red fruit.

Flow cytometry analysis was performed at Center for Agricultural Biotechnology (CAB), Kasetsart University, Kamphaeng Saen Campus. In brief, a leaf (1-2 cm) was chopped with a sharp razor blade into 1 mL quantum stain NA UV 2 (A) along with 0.05 g polyvinyl-pyrrolidone (PVP) in a plastic Petri dish. The sample was filtered through a 30 µm disposable nylon filter prior to the addition of 1 mL quantum stain NA UV 2 (B) and incubated at room temperature for 1-3 min. Mulberry leaf cv. Khunphai was used as a reference standard for the identification of the ploidy level. Intact nuclei were analyzed using a Quantum P Flow cytometer (Quantum Analysis GmbH, Germany).

Stress treatments: For the drought stress treatments, the plants were watered on the day before the treatment. On the day of treatment, excess water was removed and the pots were weighed. Leaves and fruits were harvested after exposure to drought stress by ceasing watering for 0, 5, 7 or 10 days. For the Relative Water Content (RWC) measurements, the leaves were excised, the fresh weight (Fw) was recorded and the leaves were soaked in water for at least 4 hrs at 4°C in the dark. The leaves were blotted and the turgid weight (Tw) was measured. Finally, the leaves were dried overnight at 80°C and weighed to determine the dry weight (Dw). The RWC was calculated as previously described[20].

Extraction and High Performance Liquid Chromatography (HPLC) analysis of anthocyanins: Mulberry fruit tissues harvested from both control and drought-stressed plants at different times were immediately frozen in liquid nitrogen and

then lyophilized. The lyophilizate was powdered using a mill and stored at -80°C until analysis. The mulberry fruit powder (40 mg) was extracted using 300 µL of methanol containing 1% hydrochloric acid with ultrasonication. After centrifugation, chloroform and distilled water were added and mixed thoroughly. The mixture was sonicated in cold water for 15 min and centrifuged at 12,000 rpm for 5 min. Then, the supernatant was filtered through a 0.2 µm syringe filter prior to HPLC analysis.

The HPLC analysis of anthocyanins was performed using a Perkin Elmer liquid chromatograph equipped with a diode array (Flexar PDA UHPLC Detector). The anthocyanins were separated on a 4.6×250 mm, 5 mm particle size, C18 column (Thermo Scientific, USA) using two gradients, namely A consisting of ultrapure water including 0.1% Trifluoroacetic Acid (TFA) and B consisting of 100% acetonitrile as follows: 100% A/0% B for 2 min, 88% A/12% B for 8 min, 74% A/26% B for 7 min, 70% A/30% B for 7 min, 60% A/40% B for 6 min, 40% A/60% B for 5 min and 0% A/100% B for 5 min. The flow rate was set at 1.0 mL min^{-1} and the injection volume was 20 µL. The anthocyanins were monitored at a wavelength of 520 nm. Three independent biological replicates were measured for each sample. Cyanidin-3-glucoside (C3G) and malvidin-3-glucoside (M3G) were identified by comparisons of retention times and UV spectra with authentic standards (Sigma-Aldrich, St. Louis, MO, USA) and were quantified with their respective standard curves using linear correlation coefficients greater than 0.999.

Determination of free radical scavenging activities

DPPH scavenging activity: Analysis of the 2,2-diphenyl-2-picrylhydrazyl (DPPH)-radical scavenging activity of the fruit extracts was carried out as previously described[18]. In brief, 0.5 mM DPPH was dissolved in 10 mL of 100% ethanol. The samples were concentrated before the DPPH assays by a sample concentrator. The anthocyanin extracts were diluted to different concentrations (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mg mL^{-1}) using ethanol. To prepare the samples, 50 µL of mulberry fruit extract was mixed with 50 µL of DPPH solution in a 96-well microplate. Then, the samples were allowed to react at room temperature in the dark for 30 min. The absorbance of the reaction solution was measured spectrophotometrically at 517 nm to check the reduction of DPPH. L-ascorbic acid was used as the positive control. All measurements were performed in triplicate (n = 3). The half-maximal inhibitory concentration (IC_{50}) of each sample was calculated using a linear equation from the standard curve of anthocyanin concentrations.

Evaluation of DNA protection activity: The inhibitory effect of the mulberry crude extract on supercoiled DNA breakage was assessed using the agarose gel electrophoresis method[21]. Plasmid DNA as pET28b (150 ng) was mixed with 3 µL of water and 2 µL of 50 µM $FeSO_4$, to which was added 5 µL of anthocyanin extract from mulberry fruit and 2 µL of 3% H_2O_2. Then, the mixture was incubated at 37°C for 30 min. Next, 3 µL of 6×DNA loading dye was added immediately to end the reaction. The samples (with a final total volume of 18 µL) were loaded into the gel and electrophoresis was performed at 100 V for 25 min. Finally, the reactions were visualized in 1×TAE buffer denaturing 1% (w/v) agarose gel with RedSafe™ Nucleic Acid staining (Intron, Korea).

RNA isolation and cDNA synthesis: Total RNA was extracted from different tissues (0.5 g) as previously described[22] with some modifications. The RNA quality was evaluated by both the Optical Density (OD) value (1.8-2.1) at 260/280 nm (NanoDrop1000C, Thermo Scientific) and by electrophoresis in a 1% agarose gel with sharp, clear 28s and 18s rRNA bands stained by RedSafe™ Nucleic Acid staining. The qualified RNA was preserved at -80°C.

The cDNA was synthesized using a reagent kit (Thermo Scientific, USA) following the manufacturer's instructions. Total RNA (1 µg) was mixed with 1 µL of Oligo $(dT)_{18}$ primer. Then, nuclease-free water was added to form a final volume of 12 µL. The RNA template was mixed gently, centrifuged briefly and incubated at 65°C for 5 min. Then, it was put on ice for 5 min before spinning down. After that, 4 µL of 5×reaction buffer, 1 µL of RiboLock RNase inhibitor (20 U $µL^{-1}$), 2 µL of 10 mM dNTP mix and 1 µL of RevertAid M-MuLV RT (200 U $µL^{-1}$) were added and then mixed gently and centrifuged briefly. The reaction had a final total volume of 20 µL. After that, it was incubated for 60 min at 42°C and was terminated by heating at 70°C for 5 min. The cDNA sample was stored at -20°C until use.

Quantitative real-time PCR: Quantitative RT-PCR was performed in the CFX Connect Real-Time PCR Detection system (Bio-Rad, USA) using iTaq Universal SYBR Green Supermix (Bio-Rad, USA) following the manufacturer's instructions. The PCR procedure was set with three-step cycling conditions: 3 min pre-denaturation at 95°C, followed by 40 cycles of 15 sec denaturation at 95°C, 30 sec annealing at 55°C and 30 sec polymerization at 72°C. All relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method[23]. The results were finally expressed as the absolute ratio between the number of copies of the target gene and the

Table 1: Primer sequences of the anthocyanin biosynthetic genes used in the qRT-PCR

Gene	5'-3' primer sequence	Product size (bp)
PAL (F)	CCATGGTGCTTTTCGATGCC	176
PAL (R)	GCTGTCATCCAAAATGTGTTCC	
CHS (F)	AACGGCACATGAGGTTGACG	172
CHS (R)	ATTTTTGATTTCGGTTGGCCCC	
ANS (F)	CCGATCGAGGAGAAGGAGAA	182
ANS (R)	TTGTAGTCAGTTGGTGTCTTGG	
Actin (F)	CCTTCCCCATGCTATTCTCC	186
Actin (R)	TCTTGGCAGTCTCGAGTTCC	

bp: Base pair, F: Forward, R: Reverse, *PAL*: Phenylalanine ammonia lyase, *CHS*: Chalcone synthase, *ANS*: Anthocyanidin synthase

number of copies of the housekeeping gene (*Actin*). One-way ANOVA was conducted to evaluate the statistical significance. Primer sequences were used for qRT-PCR are listed in Table 1.

Statistical analysis: All experiments were carried out in triplicate and the results were presented as the mean±SE. Statistical analysis were performed using the GraphPad Prism 7 software. Significant differences between the treatments were determined using Tukey's HSD post hoc test.

RESULTS AND DISCUSSION

Effects of drought stress on mulberry growth: Pre drought-stressed mulberry cv. Kamphaeng Saen-MB-42-1 was used as a model to study drought responses showed Fig. 1a. Most of the cultivated mulberry plants are diploid (2n = 28 chromosomes), however natural polyploids have also been reported[24] and the total anthocyanin content increased as the ploidy level increased[25]. Flow cytometric analysis indicated that the mulberry plant used in this study was diploid (2n = 2x = 28). As shown in Fig. 1b, the large peak appeared in the flow cytometric histogram was probably the peak caused by the 2C nuclei, which contain the basic amount of the nuclear DNA of a diploid plant. Similar peak pattern was also observed for diploid mulberry shown in the previous reports[26,27]. The plants were then subjected to drought for 0, 5, 7 or 10 days in Fig. 2a. Changes in soil-water status were monitored by measuring pot weights to ensure that all plants were exposed to the same drought stress in Fig. 2b. During the imposed drought, the loss of soil-water was reflected in the decline of pot weight. However, for the well-watered plants (controls), almost no changes in pot weight were observed. The leaf RWC, which is an important indicator of water status in plants, was also measured. The mean RWC of the mulberry plants remained constant at approximately 80% during the first 3 days of simulated drought and then decreased progressively to approximately 65 and 18% after 5 and 7 days of simulated drought, respectively showed in Fig. 2c.

Anthocyanin accumulation in response to drought stress: Simple and rapid HPLC was used for the separation and determination of the anthocyanins. Anthocyanins are found in the cell vacuoles, mainly in the flowers and fruits but also in the leaves, stems and roots. Cyanidin-3-glucoside (C3G), the most common anthocyanin in mulberry fruits[28] and malvidin-3-glucoside (M3G) levels were monitored over the drought period. Even though the M3G content was relatively lower than for C3G, the amounts of both compounds began to increase after 7 days of drought in Fig. 3a and b. On day 10, the C3G and M3G levels in the mulberry fruits increased 3.5- and 5-fold, respectively, compared to the control. Commercially available C3G and M3G standards were used for HPLC quantification in Fig. 3c. A representative chromatogram of the sample was shown in Fig. 3d. This result suggested that C3G and M3Glevels were significantly enhanced in response to drought stress. However, neither compound was detected in leaves using the HPLC method.

Drought stress leads to the production of secondary metabolites that involved in plant defense response[29]. Previous study demonstrated that flavonoids, a class of polyphenolic secondary metabolites, can mitigate drought[30]. In addition, the phytohormone Abscisic Acid (ABA) also plays a key role in abiotic stress tolerance in plants. The ABA levels increase in response to drought, acting as a regulator of plant water balance[31-33]. Furthermore, ABA and sucrose synergistically enhanced anthocyanins in leaf discs of the grapevine (*Vitis vinifera* L.)[34]. In bilberry fruits, exogenous ABA treatment significantly promoted anthocyanin biosynthesis, whereas the ABA biosynthesis inhibitor, fluridone, delayed anthocyanin accumulation[35]. Drought-stressed plants accumulate anthocyanins, possibly due to osmotic regulation and increased ABA content, thus suggesting that these metabolites can be used as positive markers for drought stress[36,37].

Expression profiling of genes involved in anthocyanin biosynthesis: During fruit developmental stages, the expression was investigated of three anthocyanin biosynthesis genes (*PAL*, *CHS* and *ANS*) in mulberry by RT-qPCR in Fig. 4a. The *PAL* is the enzyme involved in the first step of phenyl propanoid biosynthesis[38]. The *CHS* and *ANS* belong to Early

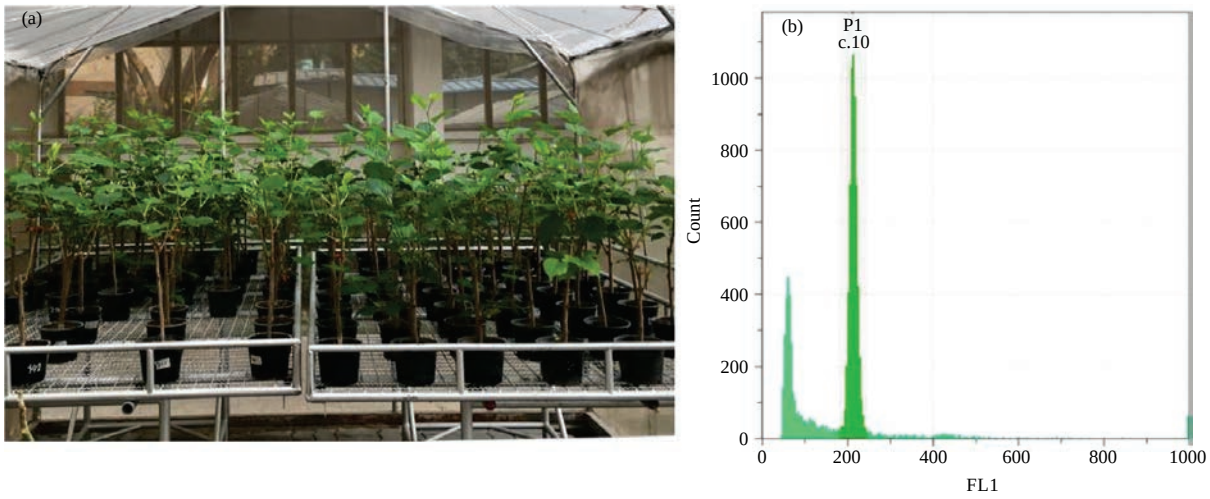

Fig. 1(a-b): General view of mulberry

(a) Representative image of pre drought-stressed plants and (b) Histogram of relative nuclear DNA content from fresh leaves

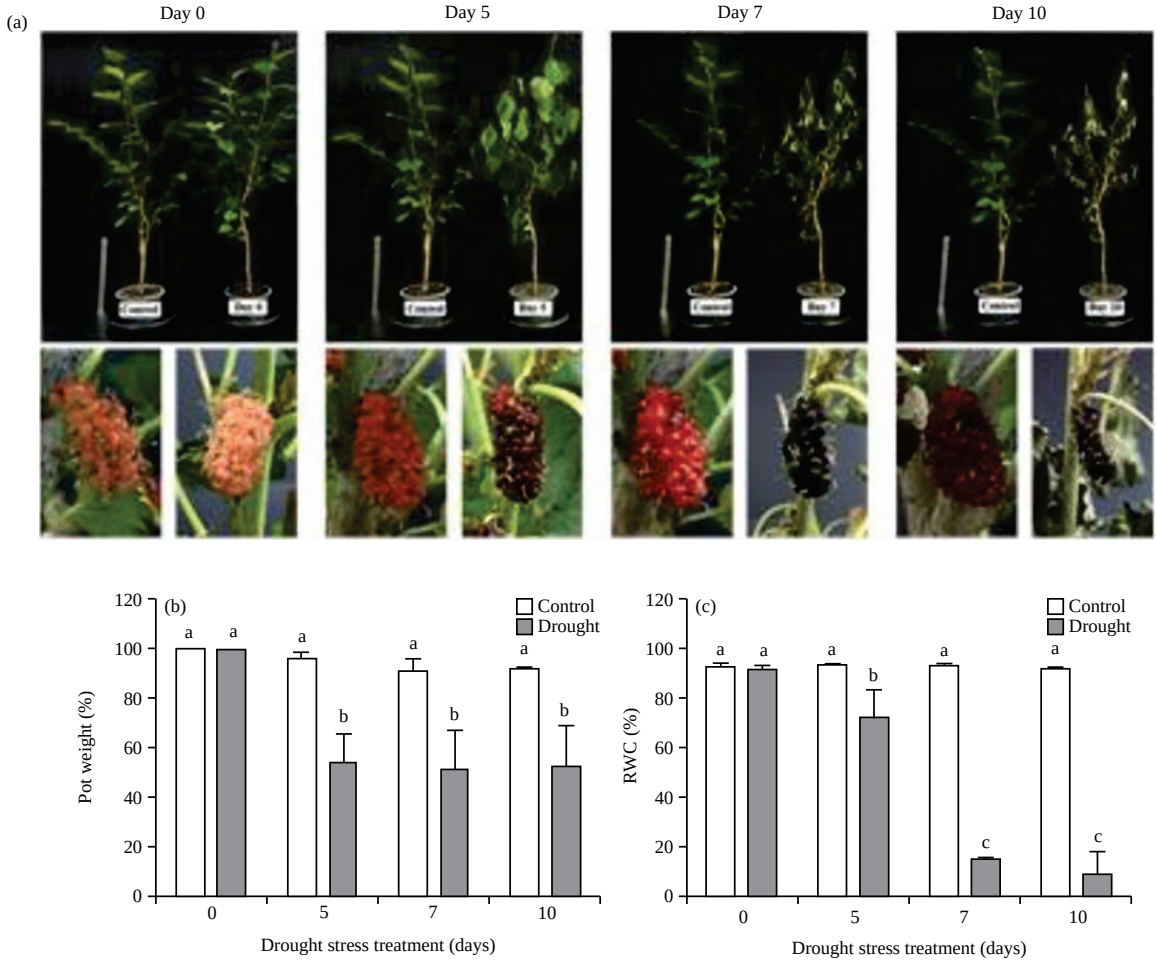

Fig. 2(a-c): Mulberry plant stress response

(a) Phenotypes of plant after exposing to drought far 0, 5, 7 or 10 days, (b) Changes in pot weights over a drought period, (c) Relationship between relative water content and drought stress exposure. Differences between day 0 and different lowercase letters above columns are significantly ($p<0.05$) different. Standard error bars calculated from results of 5-6 biological replicates

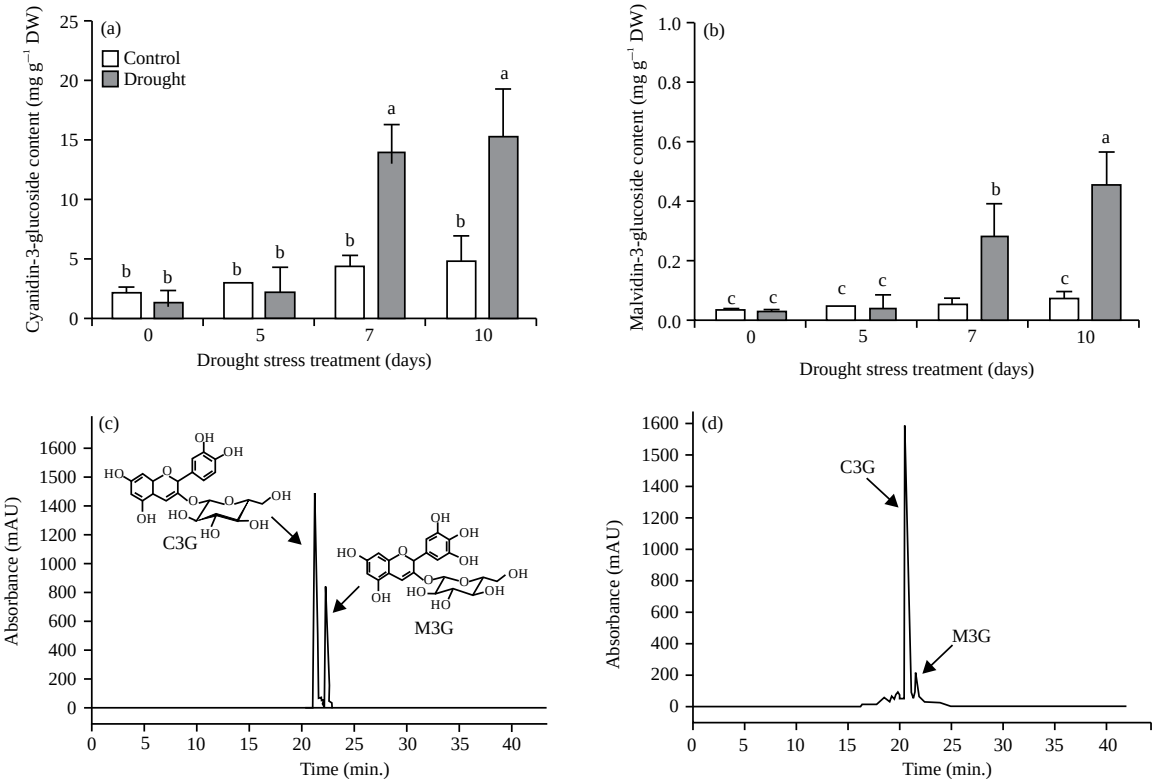

Fig. 3(a-d): Anthocyanin accumulation during drought

(a) Cyanidin-3-glucoside content in mulberry fruit, (b) Malvidin-3-glucoside content in mulberry fruit, (c) Representative HPLC chromatograms showing the peaks and retention times of cyanidin-3-glucoside and malvidin-3-glucoside used as standards and (d) Representative HPLC chromatograms the sample. Differences between day 0 and the other treatments analyzed using two-way ANOVA were statistically significant ($p<0.05$). The error bars calculated from results of 3-5 biological replicates

Biosynthesis Genes (EBGs) and Late Biosynthesis Genes (LBGs), respectively[39,40]. The expression of *PAL* was lower in the leaf, compared to the other tissues in Fig. 4b. The early biosynthetic gene *CHS* was highly expressed at the ripening stage in Fig. 4c. The transcript levels of *ANS* in particular were very low at the beginning of fruit development and strongly increased upon ripening showed in Fig. 4d. On the contrary, no induction was observed in *PAL* gene expression during fruit development.

Consistent with previous reported data, the tissue-specific expression analysis in mulberry indicates that the expression of *CHS* and *ANS* genes were well-correlated with the anthocyanin accumulation during fruit development[41]. Similar results have been reported in many plants including grape[11], Chinese bayberry[42], cauliflower[43], kiwifruit[44] and blackberry[45].

Expression of anthocyanin biosynthetic genes in response to drought: The increase in anthocyanins in response to drought raises the question of how biosynthesis is regulated. The result of RT-qPCR shows that the expression of *PAL* was induced after 5 days of drought, then decreased gradually in Fig. 5a. Nevertheless, an increasing tendency was observed for *CHS* and *ANS* transcripts after drought exposure. Especially, the expression of *CHS* was more than 5-fold increased on day 10 when compared to the controls in Fig. 5b and c.

The increased anthocyanin concentration observed in the present study might have been due to the up-regulation of the structural genes, *CHS* and *ANS*, which are involved in anthocyanin biosynthesis. The expression of the *CHS* and *ANS* genes under drought appeared to be positively linked with the anthocyanin contents as also found in wheat, potato and the medicinal plant, *Scutellaria baicalensis*[46-48]. In addition, *ANS* is one of the key enzymes in the late stage of biosynthesis pathway which may determine the kinetics of anthocyanin production[48]. Nevertheless, the expression of regulatory genes should be further investigated to clearly understand the anthocyanin biosynthesis pathway in mulberry fruits during drought. The increase in the anthocyanin content might have been caused by the regulatory genes that control the structural gene expression[49,50].

Antioxidant and DNA damage protective activities of mulberry fruit extracts: The antioxidant activities of mulberry fruit extracts were evaluated using DPPH assay with

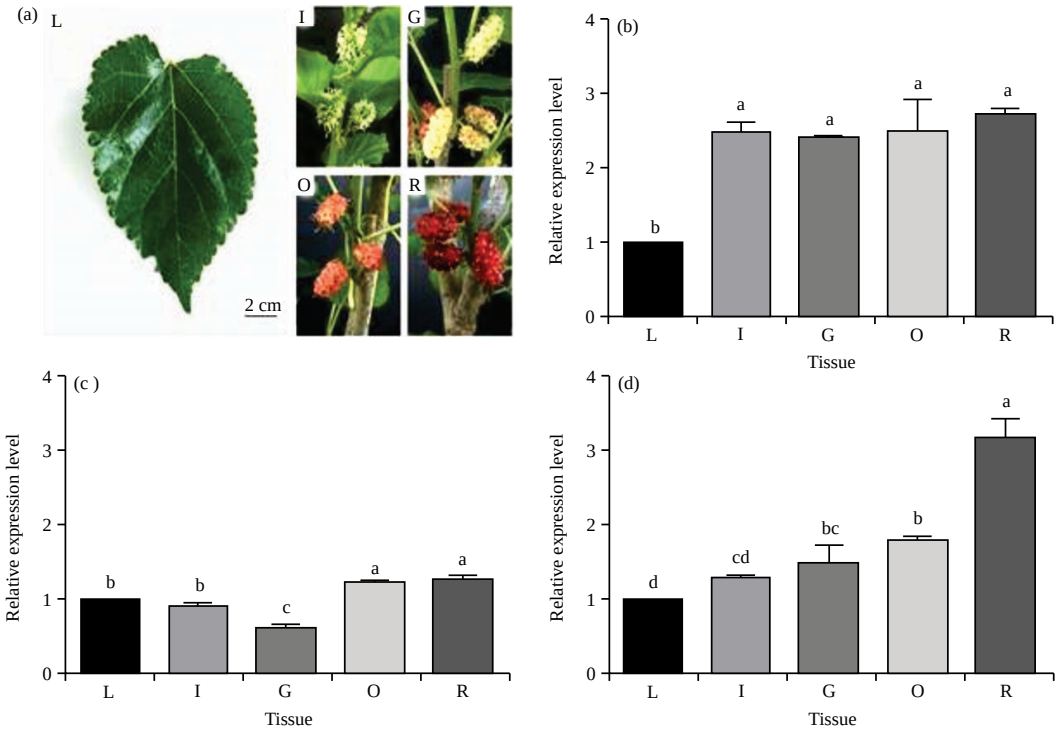

Fig. 4(a-d): Tissue-specific expression patterns of anthocyanin biosynthetic genes in various tissue

A: Phenotypes of the mulberry tissue, L: Leaf, I: Inflorescence, G: Green fruit, O: Orange fruit and R: Red fruit, B: qRT-PCR was used to analyze the expression levels of *PAL*, *CHS* and *ANS*. The expression level was normalized to leaf sample. Actin was used as an internal control

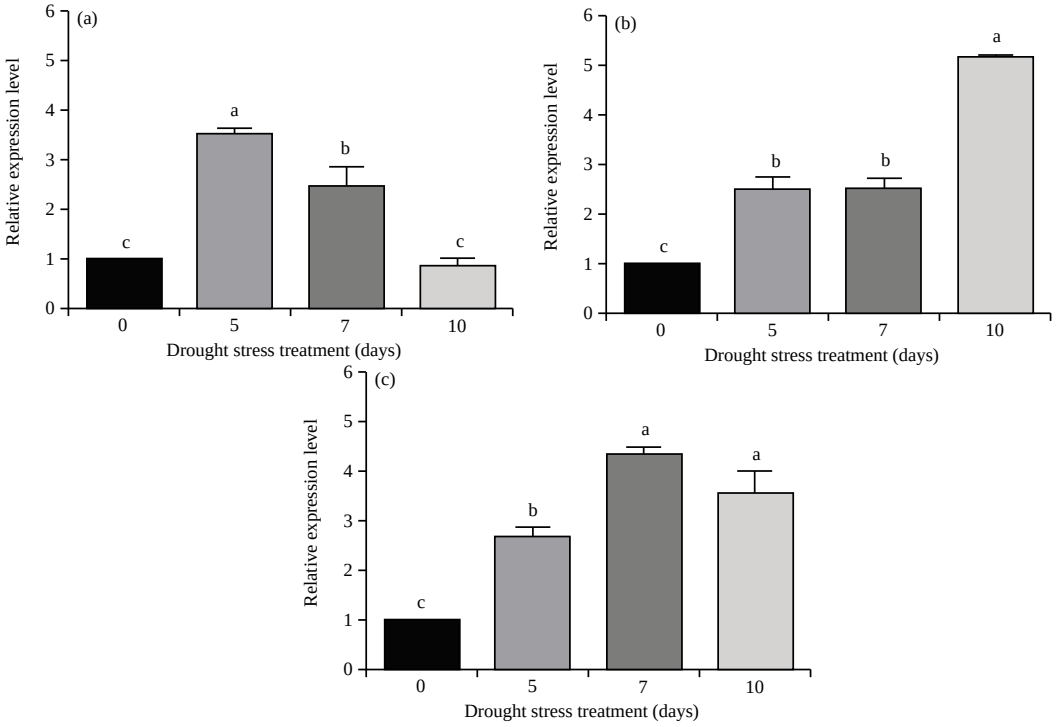

Fig. 5(a-c): Expression profiles of 3 anthocyanin biosynthetic genes in fruit tissue under drought stress

qRT-PCR was used to analyze the expression of (a) *PAL*, (b) *CHS* and (c) *ANS*. Actin was used as an internal control

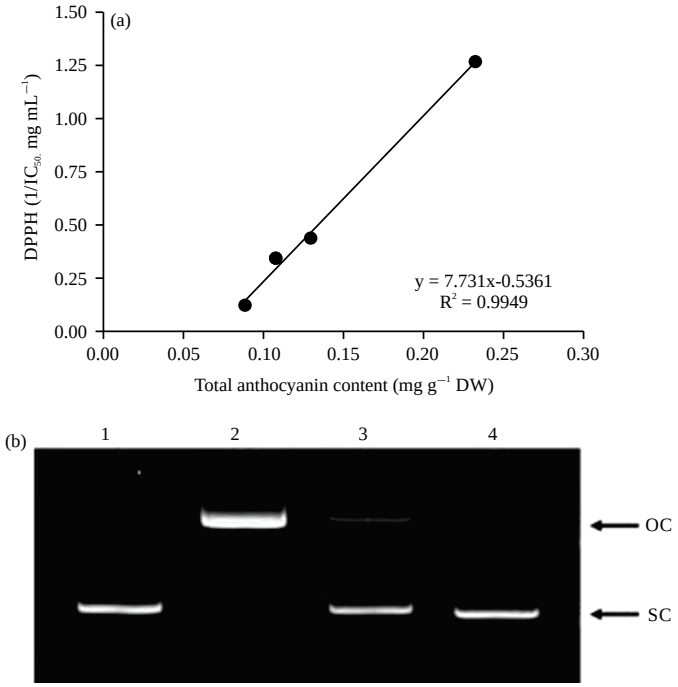

Fig. 6(a-b): Antioxidant and DNA protective efficacy of mulberry fruit extract

(a) Correlation between total anthocyanin content and antioxidant activity, (b) Electrophoretic pattern of plasmid DNA after oxidative damage induced bt Fenton's reagent Lane 1, control, Lane 2, DNA incubated with Fenton's reagent and Lanes 3 and 4, DNA incubated with Fenton's reagent in the presence of 2.5 and 5 mg mL^{-1} anthocyanin extracts, respectively

ascorbic acid as a standard. This method is widely used for determining the ability of compounds to act as free radical scavengers[51]. A lower IC_{50} value indicates a strong antioxidant property. The fruit extracts from drought day 10 samples showed higher antioxidant activities than the other extracts which could be related to their anthocyanin content. As shown in Fig. 6a, positive correlation between the increased anthocyanin content during drought and antioxidant activity was observed (R^2 = 0.99). Therefore, the increase of anthocyanin content most likely contributes to the higher DPPH•-scavenging activity of mulberry fruits.

Overproduction of Reactive Oxygen Species (ROS) is involved in many diseases, including cardiovascular, neurodegenerative diseases, diabetes, cancer, bacterial and viral infections[52,53]. Antioxidants play a crucial role in protecting against DNA damage caused by ROS[54]. However, there is still lack of studies reporting on the protective activity of mulberry fruit extracts against DNA damage. The approach used in the present study was based on DNA breakage induced by H_2O_2, a major form of ROS. In the absence of ROS, double-stranded plasmid DNA has a Supercoiled Conformation (SC). When the DNA is bound by free radicals, the DNA strand is broken, resulting in an Open-circular (OC) form. The results revealed the protective effect of mulberry fruit extracts on DNA damage as indicated by an increase in the SC form (77 and 89% at 2.5 and 5.0 mg mL^{-1} extracts, respectively) compared with the negative control showed in Fig. 6b.

There is now increasing interest in the antioxidant properties of phytochemicals which are involved in chronic disease prevention[55-57]. In *Lactuca sativa*, C3G has been shown to scavenge superoxide radicals generated by chloroplasts, suggesting that anthocyanins can serve as effective antioxidants[58]. The potential of plant extracts to prevent DNA damage has been reported in several studies[54,59,60]. However, the present study is the first published on the DNA protective activity of mulberry fruit extracts. The results were consistent with previous research suggesting that anthocyanins protect DNA against oxidative damage by forming co-pigmentation complexes that shield the DNA from H_2O_2[61,62].

CONCLUSION

A number of environmental conditions, including low temperature, UV radiation, wounding and pathogen attacks are known to activate the expression of genes involved in the general phenylpropanoid metabolism. Drought stress is

always considered a negative factor responsible for severe yield losses in agriculture. However, some drought stress can lead to the enhancement of secondary metabolite production. The present report is the first published on drought-induced anthocyanin accumulation in mulberry fruits. These results implied that environmental stimuli (drought in this case) can be applied during cultivation to increase the anthocyanin content. Furthermore, the gene expression data presented here provide important information on key anthocyanin biosynthetic genes, which could be useful for the production of mulberry with enhanced health and nutritional benefits in the future.

SIGNIFICANCE STATEMENT

This study showed the enhancement of anthocyanin production in mulberry fruits by applying decreased irrigation. Results demonstrated that cyanidin-3-glucoside and malvidin-3-glucoside contents significantly increased after 10 days of drought and can be used further. The up-regulation of structural genes in anthocyanin biosynthesis was also observed in response to drought stress. Moreover, the increase in anthocyanin content was correlated with the antioxidant properties of the fruit extracts.

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