

THE TEMPORAL CHANGES OF PIGMENTS CONTENT AND KEY ENZYME ACTIVITIES DURING AUTUMNAL TURNING PERIOD OF *Pistacia chinensis* BUNGE

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ABSTRACT

In this study, the temporal regularity of pigments and key enzyme intermediates of *Pistacia chinensis* Bunge in the color-changing period was investigated to provide a theoretical basis for exploring the mechanism of leaf discoloration. The pigment content and activities of key enzymes of *P. chinensis* during leaf discoloration were investigated. The correlation between leaf discoloration and environmental factors (temperature, relative humidity, light) was also analyzed. During the color change, the chlorophyll content decreased, while no significant change in the carotenoid content was observed. The anthocyanin content significantly increased in the middle of the period of color change. The ratios of carotenoids/chlorophyll and anthocyanins/chlorophyll showed an upward trend during the period of color change. The lightness parameter (*L*) and hue parameter (*B*) of *P. chinensis* Bunge leaves showed a fluctuating tendency, reaching the highest value at the beginning of color conversion. The hue parameter (*A*) showed an upward trend at the color conversion stage. The 5-aminolevulinic acid (ALA) and porphobilinogen (PBG) values showed an upward-downward-upward trend. The contents of ALA and PBG at the end of the color transformation were 2.01 times and 2.88 times higher than those at the beginning. The activity of phenylalaninammonio-lyase increased during the color change period. Chalcone isomerase and chalcone synthase first increased, then declined, reaching their highest level in the middle of color conversion, 261.0 u/g and 157.3 u/g, respectively. Although the activities of both enzymes declined at the end of the color conversion, they were higher than at the beginning of the color change. Anthocyanin content was negatively correlated with temperature, relative humidity, day length, and chalcone isomerase, whereas it was positively correlated with phenylalaninammonio-lyase. The results revealed the reasons for the discoloration of *P. chinensis* leaves in autumn and thus should be considered when exploring the mechanism of color-changing plants and performing color-changing plant applications.

Key words: color change period, essential enzymes, leaf color parameters, pigment, *Pistacia chinensis* Bunge

INTRODUCTION

Color-leafed plants with bright and colorful viewing characteristics have been widely used in the construction of urban gardens in recent years [Luo et al. 2013]. The mechanism of leaf coloration is highly complex, as it is influenced by environmental and

genetic factors [Zhang et al. 2017]. Research focuses primarily on climate conditions and critical enzymes involved in leaf color change. The direct causes of leaf color changes are related to pigment changes in type, content, and distribution [Zhang et al. 2017].

Additionally, leaves' cellular structure, physiology, biochemistry, and metabolism processes are modified during the period of color change [Lev-yadun et al. 2012, Bwcker et al. 2014, Li et al. 2016].

Pistacia chinensis Bunge is a broadleaf species of the Anacardiaceae family. Its leaf color turns deep red in autumn and is valuable for enjoying the sight of leaves, flowers, and fruits. It is also a unique ornamental tree species with valuable arbor decoration forest [Hu et al. 2010]. Some studies have focused on the relationship between pigment content, leaf color parameters, and leaf color change with carbohydrates of *P. chinensis* [Hu et al. 2010, Guo et al. 2017]. For plants with colored foliage, environmental factors such as light, temperature, and air humidity greatly influence the formation of colored leaves during the period of color change [Li et al. 2017]. However, the relationship between these environmental factors, pigment metabolic intermediates, essential enzymes, and leaf color has not been previously studied. This study was therefore undertaken to examine the temporal dynamics, regulation of pigment, and critical enzyme activities during leaf discoloration of *P. chinensis*. The relationship between environmental factors and leaf discoloration was also investigated. This study provides a scientific theoretical basis for exploring the color-changing mechanism of *P. chinensis* Bunge and improving the leaf color of this tree species. It also lays the foundation for selecting and cultivating the local excellent *P. chinensis* Bunge.

MATERIALS AND METHODS

Plant materials. *P. chinensis* leaves were harvested at the Shanxi Agricultural University forestry station (112°28'E, 37°12'N). This region has a temperate monsoon climate with an average annual temperature of 13°C, an average annual rainfall of 458 mm, and an altitude of 1914 m. This region also has the characteristics of less snow and dry-cold in winter, hot and rainy in summer, clear and crisp days in autumn, and dry and windy in spring.

Fifteen well-grown and sturdy *P. chinensis* trees were selected, and every 5 plants were repeated 3 times. Plant materials were sampled every seven days from October 1, 2020, to November 12. Healthy leaves of similar size were selected from each tree (Fig. 1). The concentration of the photosynthetic pigments and anthocyanins was determined immediately.

Climate data (temperature, relative humidity, and day length) were collected for the sampling period from October 1 to November 12, shown in Figure 2. Climate data were observed from the Tianqi Intelligent Ecological Station in Shanxi Agricultural University's forestry station.

Leaf color parameters. The leaf color parameters were determined following the method of Li et al. [2017]. Following sampling, the leaves were shot close-up with a camera. Then, we adopted the Lab mode to determine the value of leaf color parameters *L*, *A*, and *B* sheet color settings in Adobe Photoshop

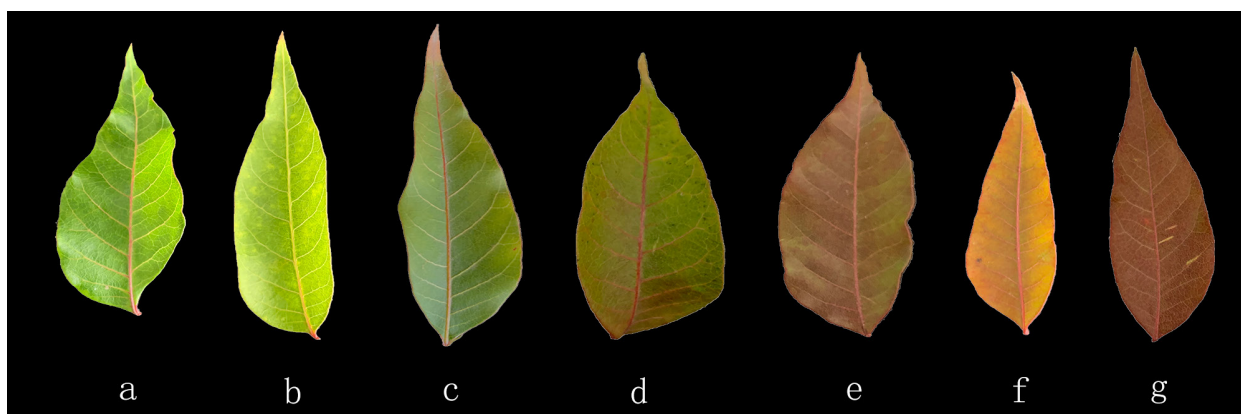


Fig. 1. a, b, c, d, e, and f were leaf colors observed on October 1, October 8, October 15, October 22, October 29, November 5, and November 12, respectively

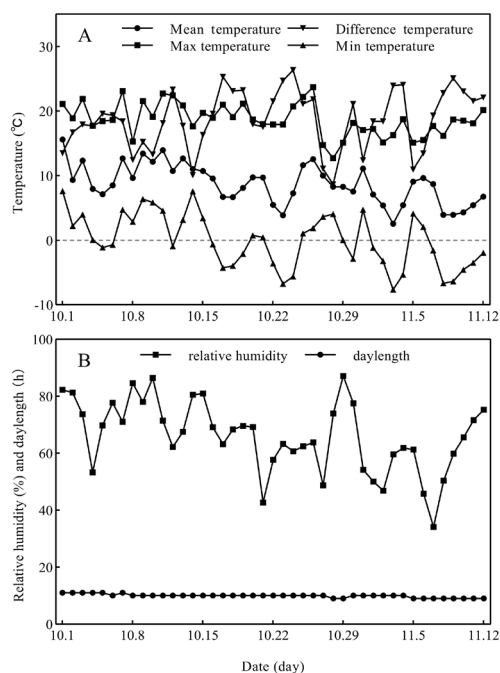


Fig. 2. Changes in temperature, relative humidity, and day length during the experimental period

CS6 software. The higher the L value is, the higher the light level. A value changes from negative to positive, and the color changes from green to red. When the value of A is greater, the red is deeper; when the A value is smaller, the green is deeper. The B value changes from negative to positive, and the color changes from blue to yellow. When the B value is greater, the yellow color is more profound; the blue color is deeper when the B value is smaller. We selected the leaves randomly for each date. We picked 4 points evenly in the leaves to avoid the leaf veins. Finally, the average leaf color parameters of the 4-dot sheets were obtained.

Determination of photosynthetic pigments and anthocyanin content. The photosynthetic pigment content was determined according to Li Li and Zhang [2016]. Three fresh *P. chinensis* leaves without petioles and veins, 0.1 g. The leaves were placed into a test tube, fixed to a volume of 5 ml with 95% ethanol, and incubated in the dark for 24 h at room temperature. After the leaves turned white, the supernatant was taken, and the absorbance value (OD) was measured at 665 nm, 649 nm, and 470 nm with a spectropho-

tometer. Anthocyanin was quantified using the hydrochloric acid extraction method of Cai [2017]. One gram of fresh *P. chinensis* leaves were weighed and placed in a test tube, 10 ml of 0.1 mol/L HCl was added, and the test tube was placed in a 32°C incubator and soaked for at least 4 h. The supernatant was taken, and the OD value was read with a spectrophotometer at a wavelength of 530 nm. Please briefly describe each method used.

Chlorophyll metabolite content. δ -aminolevulinic acid (ALA) was quantified using a method by Wang et al. [1997]. A total of 0.4 g of *P. chinensis* leaves was weighed, 4 ml of 4% trichloroacetic acid was added, and the solution was centrifuged at 12000 g for 10 min. Took 1 ml of 4% trichloroacetic acid, added 1 mol/L NaAc 500 μ l and 50 μ l acetylacetone, placed in boiling water bath for 10 min, cool to room temperature, and then centrifuged under 12000 g for 10 min, took 1.5 ml of supernatant, added 1.5 ml of Ehrlich Hg reagent, stood in the dark for 15 min, and then used a spectrophotometer to measure the OD value at 553 nm. Porphobilinogen (PBG) was quantified using a method by Xu et al. [2006]. We weighed 0.2 g of *P. chinensis* leaves, added liquid nitrogen to the leaves, fully ground them, added 4 ml of extraction buffer (0.6 mol/L Tris, 0.1 mol/L EDTA, pH = 8.2), fully homogenized them, centrifuged for 10 min at 12000 g, took 1.5 ml of supernatant, added 1.5 ml of Ehrlich Hg reagent, and after standing in the dark for 15 min, measured the OD value at 553 nm with a spectrophotometer. Please briefly describe each method used.

Anthocyanin metabolism enzyme activity. Phenylalanine ammonialyase (PAL) activity was quantified using Cai's method [2017]. *P. chinensis* leaves (0.5 g) were weighed, 1.5 ml of precooled 7 mmol/L mercaptoethanol boric acid buffer, excessive polyvinylpyrrolidone and a small amount of quartz sand were ground into a slurry in an ice bath, and then 3.5 ml of precooled extract was added to a final volume of 5 ml. The samples were centrifuged at 12 000 g and 4°C for 15 min, and the supernatant was the crude enzyme solution. Crude enzyme solution (0.1 ml), 2 ml of 0.1 mol/L boric acid buffer (pH = 8.8), and 1 ml of 0.02 mol/L L-phenylalanine solution were mixed with a vortex mixer, and the initial absorbance value

at 290 nm was immediately measured with a spectrophotometer. The measured tubes were kept in a water bath at 30°C for 30 min, and then the absorbance value of each tube was measured at 290 nm. Chalcone isomerase (CHI) and chalcone synthase (CHS) activities were quantified using the CHI kit and CHS kit (Meimian, ELISA test kit). Please briefly describe each method used.

Statistical analysis. Microsoft Excel 2016 was used for data statistics. SPSS 16.0 was used for significance discriminant analysis and regression analysis. R Studio was used for significance analysis. The differences between groups were determined using a one-way analysis of variance (ANOVA) and Duncan's new multiple-range method (Duncan). GraphPad Prism 8.0 and Adobe Illustrator CS6 were used for drawing.

RESULT

Differences in pigment content. The chlorophyll a, b, and total chlorophyll content showed a downward trend over the whole period of color change (Fig. 3), showing significant differences among different sampling periods. The counts on October 1 were 4.6, 12.9, and 5.8 times those on November 12, respectively. The carotenoid trend was stable, but carotenoids remained below 1.192 mg/g on October 1. There was a grad-

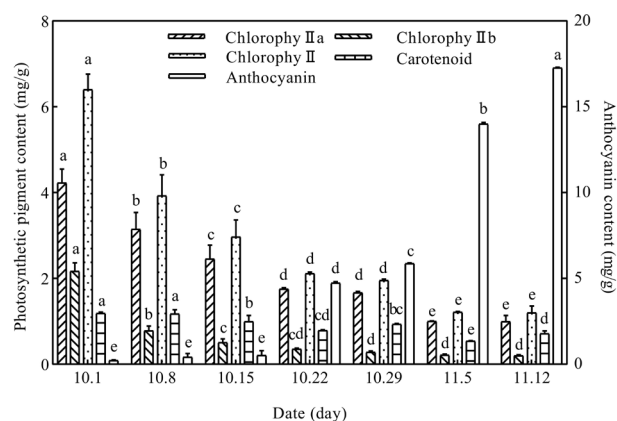


Fig. 3. Changes in pigment contents with different *Pistacia chinensis* Bunge leaf colors. The error line in the figure is the standard error of the average value. Different small letters indicate significant differences at the level of $P < 0.05$

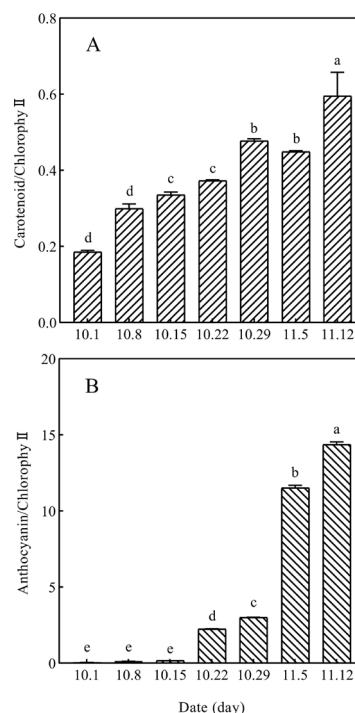
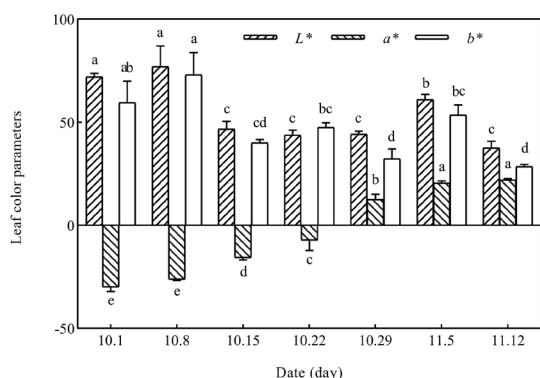


Fig. 4. Changes in pigment ratio with different *Pistacia chinensis* Bunge leaf colors. The error line in the figure is the standard error of the average value. Different small letters indicate significant differences at the level of $P < 0.05$

ual increase in anthocyanin content, which increased sharply between October 15 and 22, 9.2 times higher than in the leaf sampled on October 1. The anthocyanin content at the end of the stage was 74.1 times that of October 1. The ratios of carotenoids/chlorophyll and anthocyanins/chlorophyll increased in the color-change stage of *Pistacia chinensis* Bunge (Fig. 4).

Differences in leaf color parameters. The A value of the hue parameter continually increased during the color-change stage, and the leaves gradually changed from green to purple. The A value on November 12 was 3.4 times higher than that on October 1. The lightness parameter L and the hue parameter B showed similar changes. From October 1 to October 8, L and B showed an increasing trend and achieved the highest values of 77 and 73, respectively. After that, L and B decreased and increased substantially on November 5 (Fig. 5).



L – brightness, A – the red-green degree of the color, B – the yellow-blue degree of the color

Fig. 5. Changes in leaf color parameters with different *Pistacia chinensis* Bunge leaf colors. The error line in the figure is the standard error of the average value. Different small letters indicate significant differences at the level of $P < 0.05$

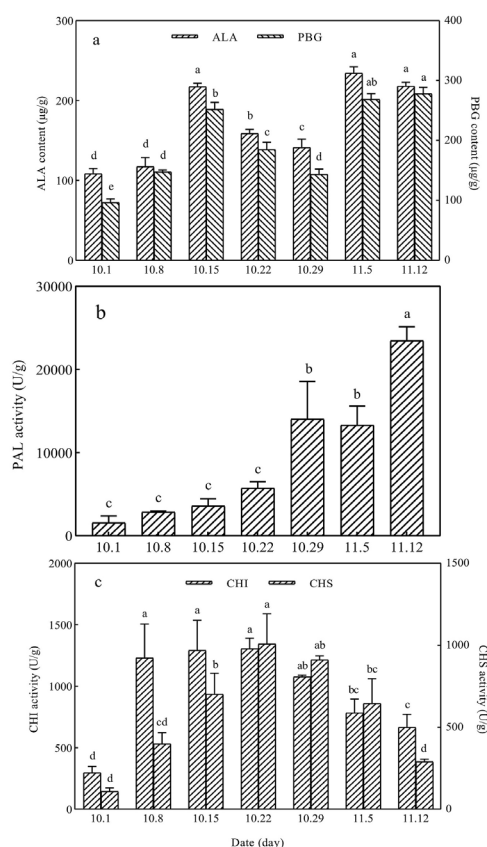
Differences in key enzyme activities. The ALA and PBG contents tended to increase-decrease-increase, but the activity of both enzymes was higher than that on October 1 (Fig. 6a). On November 12, the counts of ALA and PBG were 2.01 and 2.88, respectively, times greater than those on October 1.

PAL activity increased during the color transition period, and on October 29, a significant increase was observed relative to October 22. The activity from the color-change stage to the end was 14.9 times higher than in October (Fig. 6b). The activities of CHI and CHS first increased and then decreased in the color-change stage (Fig. 6c). During the medium color-change stage (October 22), CHI and CHS activities reached the highest levels of 1305.1 U/g and 1008.1 U/g, respectively. The activities of CHI and CHS decreased from October 22 to November 12, but the activities were still higher than those from October 1 to October 22.

Differences in correlation analysis. The correlation analysis showed that temperature, relative humidity, and day length are the main meteorological factors that affect leaf color change (Fig. 7). Temperature and day length positively correlated with CHI, whereas a negative correlation was found between temperature, relative humidity, day length, and anthocyanin. The

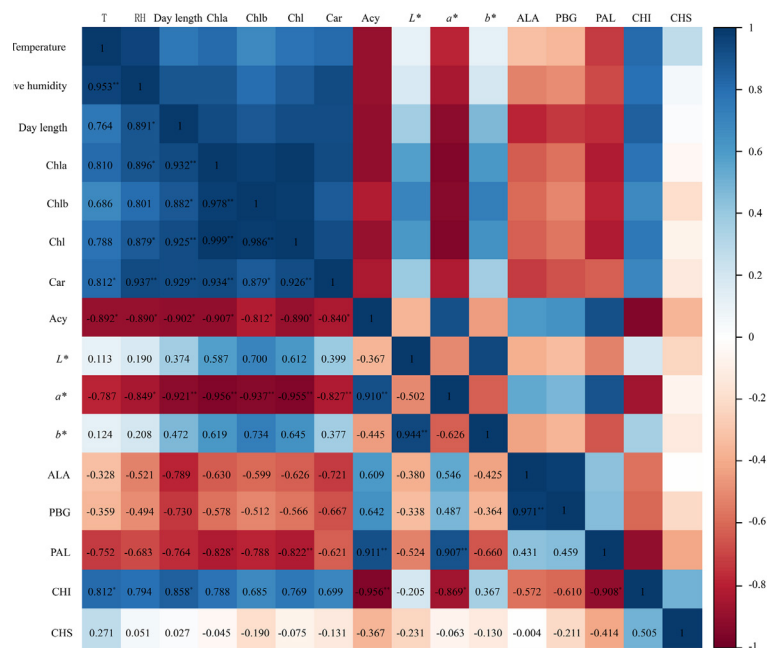
relative humidity and day length were positively correlated with chlorophyll content. Anthocyanin was also positively correlated with PAL. In addition, there was a negative correlation between anthocyanin and CHI.

Chlorophyll, carotenoids, and anthocyanins were used as dependent variables, and climate factors and key enzymes were used as independent variables. When we processed with a multivariate gradual linear regression, the regression equations were chlorophyll = $-10.07 + 1.273$ (day length; $R = 0.925$, $P < 0.01$), carotenoids = $-0.554 + 0.022$ (relative humidity; $R =$



ALA – δ -aminolevulinic acid, PBG – porphobilinogen, PAL – phenylalanine ammonia-lyase, CHI – chalcone isomerase, CHS – represents chalcone synthase

Fig. 6. Changes in key enzyme activities with different *Pistacia chinensis* Bunge leaf colors. The error line in the figure is the standard error of the average value. Different small letters indicate significant differences at the level of $P < 0.05$



T – temperature, RH – relative humidity, Chla – chlorophyll-a, Chlb – chlorophyll-b, Chl – chlorophyll; Car – carotenoid; Acy – anthocyanin, L – brightness, a – the red-green degree of the color, b – the yellow-blue degree of the color, ALA – δ -aminolevulinic acid, PBG – porphobilinogen, PAL – phenylalanine ammonia-lyase, CHI – chalcone isomerase, CHS – represents chalcone synthase

Fig. 7. The correlation between climate and physiological indexes of *Pistacia chinensis* Bung. (* $p \leq 0.05$, ** $p \leq 0.01$). Temperature, humidity, and day length are the mean values of 7 days

0.937, $P < 0.01$), and anthocyanin = $33.077 - 0.122$ (CHI; $R = 0.956$, $P < 0.01$). Chlorophyll, carotenoid, and anthocyanin were strongly linearly correlated with day length, relative humidity, and CHI, respectively. The accumulation of anthocyanins is affected by light and temperature.

DISCUSSION

Change in pigment content in the leaf color changed stage. Chlorophyll a and chlorophyll b, carotenoids, and anthocyanins are the three primary pigments in the leaves of higher plants [Jiang et al. 2005], and the change in leaf color is related to the type, content, and distribution of pigments in the leaves [Hong et al. 2010]. The change tendency in pigment content

was consistent with the leaf color of *Euonymus grandifloras*, *Cotinus coggygria*, and *Acer truncatum* in autumn [Sun and Li 2015]. The content of anthocyanins increased, while the content of chlorophylls and carotenoids decreased with time [Sun and Li 2015]. In this study, the content of carotenoids was low, but the content of anthocyanins was high in red leaves, which indicates that carotenoids were not the primary pigments in red leaves, while anthocyanins were the essential pigments affecting leaf color. Our results agree with the findings of Dai et al. [2015], who revealed that anthocyanins are the primary factor affecting the rubrum color change of *Loropetalum chinense* var. Anthocyanins have been reported as water-soluble plant pigments essential in turning red for flowers and leaves [Liu and Yu 2011]. Additionally, anthocyanins

are flavonoids containing many phenolic hydroxyl groups that are unstable in the natural state, mainly in the form of glycosides, and are found in the vacuoles of plant epidermal cells [Ge et al. 2012]. In autumn, the illumination time is shortened, the temperature is reduced, the chlorophyll begins to degrade gradually, and at the same time, it promotes the synthesis and accumulation of anthocyanins so that the type, content, and proportion of pigments in leaves change, and then the leaf color changes [Liu and Yu 2011, Wu et al. 2016]. Previously published works on *Zelkova serrata* [Zhang et al. 2015], *Euonymus europaea* [Zhuo et al. 2018], and *Davidia involucrata* [Jiang et al. 2019] demonstrated that the most direct cause for the change in leaf color was the significant increase in anthocyanin content and the change in pigment ratio. This research showed that the anthocyanin content increased significantly after October 15 and October 29, with rates of 89% and 58%, respectively, and the anthocyanin/chlorophyll ratio showed the same pattern. The correlation analysis showed a significant positive correlation between the anthocyanin content and hue parameter *A*. Accordingly, the direct cause of leaf color turning in *P. chinensis* Bunge was an increase in anthocyanin content and the anthocyanin/chlorophyll ratio.

Environmental and physiological mechanisms affect leaf color change. ALA and PBG are essential substances in the early stage of chlorophyll synthesis and have been found in leaf color mutants of various plants [Guo 2013]. Chen [2008] demonstrated that ALA synthesis was the primary step limiting the conversion rate of glutamate to chlorophyll in intact chloroplasts. ALA is a common precursor for synthesizing tetrapyrroles in living organisms: the synthesis starts with glutamic acid, produced by a 3-step reaction. Two ALA molecules catalyzed by porphobilinogen synthase and condensation reaction are converted to PBG [Luo 2020]. The chlorophyll biosynthesis process is complex, and obstacles may appear at any step. The intermediate product in front of the obstacles accumulates, and the obstacles behind it decrease. Wang et al. [1996] noted in their study of wheat chlorophyll that during the de-greening process, Uro III gradually decreased, but Uro Acute accumulated at the initiation of greening.

It indicates chlorophyll synthesis may be blocked in Uroporphyrinogen III [Wang et al. 1996, Huang et al. 2005, He et al. 2006]. Yuan et al. [2010] reported that the content of ALA was high, but that of chlorophylls was low; hence, leaf yellowing is proposed to be related to the inhibition of chlorophyll transformation in *Ligustrum vicaryi*. This study revealed that ALA and PBG levels first increased and then decreased. However, their contents were superior to the first sampling period, and ALA and PBG accumulated during the color-changed stage. In addition, the contents of chlorophyll a, chlorophyll b, and total chlorophyll were decreased, which suggests that chlorophyll synthesis was inhibited, agreeing with the findings of Cui et al. [2001] on rice mutants.

The anthocyanin metabolic pathway has been gradually understood since 1980 through color transformation studies on *Zea mays* L. and *Petunia hybrida* [Zhu et al. 2016]. Several studies have identified the key enzymes of the anthocyanin metabolic pathway: phenylalanine ammonia-lyase (PAL), chalcone isomerase (CHI), chalcone synthase (CHS), dihydroflavonol reductase (DFR), flavanone 3-hydroxylase (F3H), anthocyanin synthase (ANS), and flavonoid 3-O-glucose transferase (UGFT) [Davies et al. 2012]. Furthermore, light is the most important environmental factor affecting leaf color change by influencing the activity of pigment-related enzymes in leaves [Wang et al. 2020]. PAL, CHS, and ANS are vital enzymes in anthocyanin synthesis and thus play essential roles in forming leaf color [Liao et al. 2000, Nie et al. 2008, Jiang et al. 2019]. Anthocyanin content was positively correlated with PAL in *Anthurium* [Li 2012], and light has been found to induce PAL activity and anthocyanin content in leaves of *Prunus Cerasifera* and hence resist UV damage [Gu et al. 2015]. CHS is also a key enzyme that catalyzes the formation of chalcone from malonyl CoA and 4-coumaryl CoA in anthocyanin biosynthesis [Li et al. 2012, Guo 2013]. Therefore, CHS affects plant flower color, and CHS alteration occurs based on whether CHS expression is increased or decreased [Jiang et al. 2007]. For example, Tai et al. [2014] introduced the *CHS* gene of *Malus sinensis* into tobacco, and the anthocyanin content increased in leaves with a positive correlation between anthocyanin

content and CHS expression level. Another enzyme that catalyzes the synthesis of the anthocyanin precursor flavanone is CHI [Jiang et al. 2019], and changes in its expression level or activity have been illustrated to affect the metabolism of flavonoids such as anthocyanins and thus affect flower color [Zhou et al. 2008]. Additionally, there is a synergism between CHS and CHI, and light and ultraviolet radiation regulate their accumulation or disappearance [Shi et al. 2011]. Our study showed that PAL activity increased gradually during the color-change stage, and PAL was positively correlated with anthocyanin levels; this result is consistent with the findings reported for *Acer* [Feng et al. 2009], *Cotinus coggygria* [Nie et al. 2008], *Loropetalum chinense* var. *rubrum* [Tang et al. 2006], and *Padus virginiana* ‘Canada Red’ [Wang et al. 2008]. CHI was also positively correlated with day length and temperature and negatively correlated with anthocyanin content in the color-change stage of *Pistacia chinensis* Bunge. Moreover, CHS and CHI of *Pistacia chinensis* Bunge showed synergism, and their activities showed a comparable increase at first and then decreased.

The change in plant leaf color depends mainly on genetic factors, but the external environment also dramatically influences this process [Chalker-Scott 1999]. Low temperature can promote anthocyanin synthesis [Ougham et al. 2005], anthocyanin content increases with low temperature, and chlorophyll content positively correlates with temperature in *Acer palmatum Thunb* [Chen et al. 2010]. Low temperatures can also maintain the stability of anthocyanins in *Acer ginnala* [Hong et al. 2012]. For instance, the research carried out by Schaberg et al. [2017] demonstrated that low temperature increased the anthocyanin content by 2–10 times at the end of *Acer saccharum* growth. Additionally, anthocyanin formation in plant tissues depends on light signals, but the regulatory mechanism of light signals on anthocyanin biosynthesis is still unclear [Wang et al. 2020]. Researchers reported that chlorophyll and carotenoid content increased, whereas anthocyanin content decreased to varying degrees with differences in light periods in *Loropetalum chinense* var. *rubrum* [Fei et al. 2008] and *Zelkova serrata* [Zhang et al. 2015]. The previous results agree with our finding that chlorophyll content was positively

correlated with day length and relative humidity. On the other hand, anthocyanin content was negatively correlated with day length, temperature, and relative humidity, indicative of the importance of day length and temperature, which are the main environmental factors affecting anthocyanin accumulation and leaf coloration.

CONCLUSION

The anthocyanin content increased significantly in the middle of the color-change stage, and the direct cause for this was the anthocyanin content and anthocyanin/chlorophyll ratio. Hue parameter *A* can reflect the accumulation of anthocyanins in an indirect manner. It is also evident that anthocyanins were negatively correlated with day length, temperature, relative humidity, and CHI activity and expression but were positively correlated with PAL activity and expression. Therefore, it is concluded that the above factors strongly influence the change in *Pistacia chinensis* Bunge’s leaf color during the autumn color-change stage.

SOURCE OF FUNDING

This work was supported by the innovation and exploration of the high-quality provenance of *Pistacia chinensis*, a healthy functional tree species, Science and Technology Achievements Transformation Project of Shanxi Province and Key R&D Program of Science and Technology of Jinzhong City (Agriculture) (YZGC138; 201604D131054; Y192013).

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